University of Alberta

Regulation of Antimicrobial Mechanisms of Macrophages of the Goldfish (*Carassius auratus*)

by

Norman Frederick Neumann

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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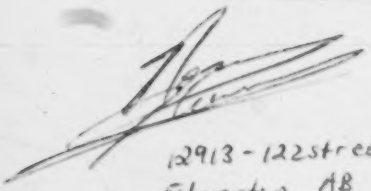

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To my wife,

BERNADETTE.

ABSTRACT

Macrophages are phagocytic leukocytes that are important mediators of host defense against infectious diseases. They are transformed into potent killer cells during infection; a process called "macrophage activation". The mechanisms that mediate macrophage activation are well defined in mammals but not in lower vertebrates. This study was undertaken to characterize the molecular mechanisms involved in the induction of antimicrobial responses of goldfish (*Carassius auratus*) macrophages.

An *in vitro* cultivation system was developed for obtaining primary macrophages from the goldfish. Leukocytes derived from the kidney hematopoietic tissues were cultured in the presence of conditioned supernatants obtained from a goldfish macrophage cell line. The proliferating leukocytes in these cultures were characterized as macrophages based on their function, morphology, cytochemistry, and flow cytometry.

Goldfish macrophages produced reactive oxygen (ROI) and reactive nitrogen (RNI) intermediates when stimulated with macrophage activating factors (MAF), derived from mitogen-stimulated goldfish kidney leukocytes. Macrophages were rapidly primed for maximal ROI responses with MAF (6 hr treatment), and the production of ROI was enhanced significantly when macrophages were co-

stimulated with bacterial lipopolysaccharide. Following maximal ROI production, activated macrophages exhibited a selective deactivation of the ROI response, but not the RNI response. These findings suggest that goldfish macrophages mount sequential antimicrobial responses, regulated by the unique kinetics of activation induced in different sub-populations of macrophages.

The molecules that induced macrophage activation were identified and purified using fast performance liquid chromatography. The following molecules were found to modulate macrophage antimicrobial responses: (1) a basic protein of 50 kD (potentially two isotypes) that induced RNI production and was responsible for the sequential induction of activated macrophage antimicrobial functions; (2) a 30 kD molecule that induced ROI production; and (3) a 12 kD molecule that down-regulated RNI production.

Understanding the mechanisms regulating the induction of macrophage antimicrobial responses will contribute significantly to our attempts to control infectious diseases in fish.

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LIST OF ABBREVIATIONS

CCM: cell conditioned medium

C-FPLC: chromatofocusing fast performance liquid chromatography

CGD: chronic granulomatous disease

cNOS: constitutive nitric oxide synthase

ConA: concanavalin A

CoQ: co-enzyme Q

DMEM: Dulbecco's Modified Eagles medium

DMSO: dimethyl sulfoxide

DPBS-G: Dulbecco's phosphate buffered saline with glucose

FPLC: fast performance liquid chromatography

GMCL: goldfish macrophage cell line

GM-CSF: granulocyte/macrophage colony stimulating factor

GP-FPLC: gel permeation fast performance liquid chromatography

HBSS: Hank's balanced salt solution

hTNF: human tumor necrosis factor

HSP: heat shock protein(s)

IDO: indoleamine 2,3-dioxygenase

IL: interleukin

IFN: interferon

iNOS: inducible nitric oxide synthase

IVDKM: *in vitro*-derived kidney macrophages

kD: kilodalton

KL: kidney leukocyte

LPS: lipopolysaccharide

MAF: macrophage activating factor(s)

M-CSF: macrophage colony stimulating factor

MGF: macrophage growth factor(s)

MW: molecular weight

NBT: nitroblue tetrazolium

N^GMMLA: N^G-monomethyl-L-arginine

NO: nitric oxide

PBS: phosphate buffered saline

PEG: polyethylene glycol

PMA: phorbol myristate acetate

RNI: reactive nitrogen intermediates

ROI: reactive oxygen intermediates

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

TNF: tumor necrosis factor

XTT: 2,3-bis[2-methoxy-4-nitro-5-sulfo-phenyl]-2H-tetrazolium-5-carboxanalide

CHAPTER I

ANTIMICROBIAL MECHANISMS OF VERTEBRATE MACROPHAGES AND THEIR INDUCTION BY CYTOKINES [1]

Introduction

Phagocytosis is the primordial defense mechanism of all metazoan organisms. Although this physiological process was first described by Haeckel in 1862, from blood cells of the gastropod, *Tethys* (Vaughan, 1965), its importance as a defense mechanism was first conceptualized by Elie Metchnikoff in 1882 (Vaughan, 1965; Beck and Habicht, 1996). Metchnikoff's classic experiment involved impaling a transparent starfish larva with a rose thorn. He noticed that amoebae-like cells invaded the damaged tissues and cavity, and surrounded the thorn; an apparent attempt by the starfish larva to defend itself against the invader. Metchnikoff immediately noticed the similarities between this primitive cellular response and inflammation of higher vertebrates. This was the beginning of cellular immunology, and provided the foundation for elucidating the roles that phagocytic cells play in defending their hosts against infection.

Macrophages are phagocytic leukocytes, essential for the maintenance of homeostasis in vertebrates. Although they are most commonly associated with host defense, these cells are also essential in tissue modeling, wound repair, and iron metabolism. Their indispensable importance within the vertebrate body can best be described by the following observations:

1) all metazoan animals possess phagocytic cells that are, in many respects, functionally reminiscent of the vertebrate macrophage;

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[1] A version of this chapter has been published: Neumann, N. F., and Belosevic, M. 1996. *Adv. Struct. Biol.*, 4: 233-254.

implicating a fundamental importance of this phagocytic cell through evolution.

2) macrophages can be found in virtually every organ, tissue, or fluid of the vertebrate body.
3) no animal models exist in which animals lack functional macrophages.

Although the term "macrophage" is commonly used to represent a specific subset of immune cells, macrophages are extremely heterogeneous and diverse populations of cells. The regulatory mechanisms orchestrating macrophage function vary considerably, depending upon the microenvironment in which a macrophage is found. For example, inflammatory sites may contain several macrophage sub-populations: uninfected resident tissue macrophages, infected macrophages, and inflammatory monocytes, each in a different developmental or regulatory state. Thus, host defense against infectious pathogens by macrophages is a dynamic process, dependent upon the orchestrated efforts of a spectrum of macrophage sub-populations and other immune cells.

The ubiquitous distribution of macrophages within the vertebrate body ensures the continuous "surveillance" of host tissues for foreign invaders. Invasive organisms are rapidly phagocytosed by macrophages, and in most cases are destroyed by lysosomal enzymes released into the phagosome. Macrophage lysosomes contain a number of degradative enzymes, including lysozyme, neutral proteases, nucleases, acid phosphatases, peroxidases, lipases, and esterases (Oppenheim and Leonard, 1989). However, several pathogenic microorganisms "parasitize" macrophages, utilizing them as a host cell for growth, replication, and/or maintenance of their life cycles (Figure 1.1). It would seem paradoxical that a pathogen would "choose" to live within an immune cell such as a macrophage. However, this paradox is easier to understand when viewed

from the parasite's perspective. Since macrophages are often the first immune cells encountered by infectious organisms, the pathogen's survival depends on their ability to prevent or circumvent lysosomal destruction by macrophages (Fig. 1.1). Thus, many parasitic infections in vertebrates, and the diseases they cause, are a direct result of the adaptations of these infectious agents to live within macrophages.

Since constitutive antimicrobial responses (i.e. lysosomal enzymes) are ineffective against destroying these intracellular pathogens, macrophages possess a repertoire of inducible cytotoxic antimicrobial mechanisms that are extremely effective in eliminating these parasites (Table 1.1). The process by which a macrophage is transformed from a normally quiescent cell into a highly potent killer cell is termed "macrophage activation". The induction of macrophage activation is initiated upon phagocytosis. Antigens from the ingested organism are processed and presented on the surface of the macrophage to other immune cells, such as T cells, which in turn secrete cytokines that signal the macrophage to destroy the phagocytosed organism. Activated macrophages produce a number of highly toxic molecules, necessitating the need for strict regulatory control of this cellular process.

Objectives of the Thesis

The functional and regulatory mechanisms orchestrating macrophage activation have been extensively characterized in mammals, while very little is known about these mechanisms in lower vertebrates, such as fish. The main objective of my thesis was to examine the molecular mechanisms involved in the regulation of inducible antimicrobial responses of fish macrophages. The specific aims of my research were :

1) to functionally characterize inducible cytotoxic responses of activated teleost macrophages.

2) to functionally and biochemically characterize macrophage activating factors involved in the regulation of inducible macrophage antimicrobial responses.
3) to assess the contributions that pathogens or pathogen-derived molecules play in initiating and triggering cytotoxic mechanisms of fish macrophages.

In the first chapter of the thesis, I review current literature pertaining to inducible antimicrobial responses of vertebrate macrophages, and how these responses are induced by cytokines. Chapter II contains a detailed description of the materials and methods used throughout the thesis. Chapter III describes results of *in vitro* cultivation techniques used to obtain macrophages for this study. Furthermore, this chapter characterizes teleost macrophages with respect to their developmental origins, morphology, cytochemistry, and functionality. The fourth and fifth chapters describe the kinetics of induction of nitric oxide production and respiratory burst responses of goldfish macrophages, respectively. In Chapter VI are the results of studies aimed at biochemically characterizing macrophage activating factors involved in the regulation of macrophage antimicrobial responses in goldfish. The final chapter (Chapter VII) is a general discussion of the findings described in this thesis. In this chapter I propose a new model of macrophage activation by which macrophage antimicrobial responses may be regulated in all classes of vertebrates, and suggest future directions of research in this area.

Literature Review

Relatively little is known about macrophage antimicrobial responses in lower vertebrates; therefore, it is important to discuss these mechanisms with respect to the well characterized mammalian macrophage. The first portion of this review focuses on the inducible microbicidal armamentarium of activated macrophages, and their induction by cytokines, as we understand them in mammals. The second portion of the review is an overview of our current understanding of these mechanisms in teleosts. The purpose of this review chapter is to highlight structurally, functionally, and comparatively the roles that macrophages play in host defense against infectious disease in vertebrates.

Antimicrobial Mechanisms of Activated Mammalian Macrophages

The Respiratory Burst

Studies as early as the 1930's demonstrated that phagocytosis was related to increased oxygen consumption by phagocytes. This "burst" in oxygen consumption was not a result of increased oxidative metabolism required by the cell for phagocytosis. Subsequent studies established a correlation between this respiratory burst and the formation of reactive oxygen intermediates, implicating the respiratory burst response as an antimicrobial mechanism of phagocytes (Iyer et al., 1961; Paul and Sbarra, 1968; Berton et al., 1984).

It is now well established that the respiratory burst is a potent antimicrobial mechanism of phagocytic cells such as macrophages, neutrophils, eosinophils and B lymphocytes (Dinauer, 1993). Although, the initial respiratory burst pathway was fully characterized in neutrophils, the

enzymatic machinery required for the respiratory burst response is similar in all phagocytes. However, the regulation of different pathway components may vary for different cell types.

The enzymes responsible for elicitation of the respiratory burst in phagocytes are collectively known as the respiratory burst oxidase or the NADPH oxidase. The respiratory burst oxidase is a multicomponent enzyme complex that, upon appropriate stimulation, assembles at the inner surface of the plasma membrane (Briggs et al., 1975). Identification of the multiple protein components comprising the respiratory burst oxidase was facilitated by two important observations: (1) that the respiratory burst response could be reconstituted in cell-free assay systems using fractionated phagocyte membranes and cytosols (Bromberg and Pick, 1984; Curnutte, 1985; McPhail et al., 1985); and (2) that individuals suffering from a genetic disorder, chronic granulomatous disease (CGD), have phagocytes that are incapable of mounting a respiratory burst response (Dinauer, 1993). Studies using purified cytosol and membrane fractions from CGD patients in cell-free assay systems demonstrated the necessity for both membrane and cytosolic protein components in the formation of the functional plasma membrane-associated oxidase (Bromberg and Pick, 1984; Curnutte et al., 1989; McPhail et al., 1985).

The first component of the respiratory burst oxidase to be identified was a low potential B-type cytochrome, termed cytochrome b558 (Segal and Jones, 1978). b558 consists of two subunits, a glycoprotein of 91 kD (gp91*phox*), and a non-glycosylated protein of 22 kD (p22*phox*) (Royer-Pokora et al., 1986; Parkos et al., 1987; Segal, 1987; Parkos et al., 1988). Three cytosolic proteins are also important for the formation of the functional oxidase: a 40kD protein, a 47 kD phosphoprotein, and a 67 kD protein (Volpp et al., 1988; Nunoi et al., 1988; Volpp et al., 1989; Leto et al., 1990; Tsunawaki et al., 1994). These cytosolic proteins exist as a complex in the cytosol (Park et al., 1992; Park et al., 1994; Finan et al., 1994), and after appropriate stimulation, translocate to the cytochrome component on the

plasma membrane to form the functional respiratory burst oxidase (Clark et al., 1990, Nauseef et al., 1991; Park, et al., 1992; Uhlinger et al., 1994). Translocation of cytosolic components to the cell membrane is mediated through the phosphorylation of multiple tyrosine residues on p47phox and p67phox, due to the actions of several different kinases, including protein kinase C (Segal et al., 1985; Rodway et al., 1990; Nauseef et al., 1991; Benna et al., 1994; Green and Phillips, 1994), protein kinase A (Chanock et al., 1994; Benna et al., 1996; DeLeo and Quinn, 1996), and mitogen-activated protein kinases (Benna et al., 1996; Deleo and Quinn, 1996). Phosphorylation of cytosolic components mediates attachment to potential cytoskeletal elements, thus facilitating and directing translocation to the phagolysosomal membrane (Quinn et al., 1989; Rotrosen et al., 1990; Heyworth et al., 1991; Nauseef et al., 1991; Woodman et al., 1991; Finan et al., 1994). The active site of the respiratory burst oxidase is orientated towards the extracellular environment and, as such, formation of reactive oxygen intermediates is directed into the phagolysosome (DeLeo and Quinn, 1996). Figure 1.2 illustrates formation and regulation of the activity of the functional respiratory burst oxidase.

The role for G-proteins in the regulation of respiratory burst function is supported by recent evidence for the requirement of Rac1 and Rac2 in formation of the oxidase (Seifert et al., 1986; Gabig et al., 1987; Ligetti et al., 1988; Abo et al., 1991, 1992; Knaus et al., 1991). Rac proteins are GTP binding proteins that are essential for generation of respiratory burst activity (DeLeo and Quinn, 1996). Rac appears to associate with an inhibitor G-protein known as Rho GDP-dissociation inhibitor, which prevents Rac from translocating to the membrane surface in resting cells (DeLeo and Quinn, 1996). Addition of purified or recombinant cytochrome b558, p47phox, p67 phox and p21rac to cell-free assay systems results in a complete respiratory burst response (Abo et al., 1992). Another G-protein involved in regulation of oxidase activity is Rap1A. Rap1A associates with cytochrome components in

the cell membrane, and may function as a membrane targeting protein for the cytochrome (DeLèo and Quinn, 1996).

Triggering of the respiratory burst in phagocytes can be initiated by receptor-mediated phagocytosis, unsaturated fatty acids, leukotrienes, chemoattractants and phorbol myristate acetate (PMA) (Badwey et al., 1981; Serhan et al., 1982; Tauber et al., 1982; Bromberg and Pick, 1983, 1984; Hartung, 1983; McPhail and Syndermann, 1983). Phagocytosis of antibody-coated pathogens via phagocyte Fc receptors elicits a very strong respiratory burst response (Wright and Silverstein, 1983; Yamamoto and Johnston, 1984; Brozna et al., 1988; Imamichi et al., 1990).

NADPH is the substrate for the respiratory burst oxidase and binds to the cytosolic protein p67phox (Smith et al., 1996). NADPH acts as a source of reducing potential for converting molecular oxygen to superoxide anion (Bromberg and Pick, 1984; McPhail et al., 1985). Production of superoxide anion ($O_2^{\cdot}$) results in the spontaneous or enzyme catalyzed production of an array of reactive oxygen products including hydrogen peroxide (H_2O_2), hydroxyl radical (OH·), hypochlorous acid (OCl-) and peroxynitrite (ONOO-) (Borregaard, 1988; Berton et al., 1988; Ischiropoulos, 1992). Hydrogen peroxide is formed by the spontaneous mutation of superoxide anion, or through an enzyme catalyzed reaction involving superoxide dismutase. Hypochlorous acid is formed in the acidic phagolysosome through the reaction of chloride ions with hydrogen peroxide in the presence of the enzyme myeloperoxidase (Borregaard, 1988). Monocytes possess the enzyme myeloperoxidase (Nichols et al., 1973) while tissue macrophages lack this enzyme (VanFurth et al., 1970). However, tissue macrophages can scavenge myeloperoxidase from exhausted neutrophils at the site of inflammation and use the enzyme for production of hypochlorous acid (Leung and Goren, 1989). Interestingly, scavenged myeloperoxidase augments the respiratory burst of tissue macrophages (Lefkowitz et al., 1993). Hydroxyl radicals are formed when hydrogen peroxide is oxidized by ferrous (Fe^{3+}) iron.

Peroxynitrite forms as a result of the spontaneous reaction of superoxide with nitric oxide radicals ($NO^{\cdot}$) produced by the enzyme nitric oxide synthase (see Fig 1.3).

Reactive intermediates generated by activated macrophages can be detrimental to the macrophage itself. Thus, macrophages have evolved mechanisms to protect themselves from their own cytotoxic molecules. Heat shock proteins (HSP), such as HSP 90, HSP 70, and HSP 60, are produced by activated macrophages, and can act as molecular sinks for reactive oxygen and nitrogen intermediates (Donati et al., 1991; Teshima et al., 1996; Hirvonen et al., 1996; Himeno et al., 1996; Szabo et al., 1996). Moreover, catabolites of tryptophan degradation, another inducible macrophage antimicrobial response (see below), can also scavenge reactive oxygen (Christen et al., 1990).

The *in vitro* antimicrobial actions of reactive oxygen intermediates formed via the respiratory burst are well documented (Table 1.2). Contribution of reactive oxygen intermediates to host defense *in vivo* is most notable in patients who suffer from CGD. These individuals readily succumb to recurrent bacterial and fungal infections (Dinauer, 1993).

The ability of pathogens to avoid initiating respiratory burst activity in macrophages has been demonstrated in many host-pathogen systems. In fact, the expression of virulence associated with a particular infection, in many cases, is associated with the ability of invading organisms to evade killing by reactive oxygen intermediates. Specific examples of the evasion mechanisms employed by pathogens are: (1) the generation of detoxifying enzymes (catalase, glutathione peroxidase, and superoxide dismutase) for reactive oxygen; (2) the use of receptors for entry into host cells whose engagement does not result in induction of respiratory burst activity (i.e. complement receptors); and (3) the production of proteins that inhibit respiratory burst oxidase assembly and function.

Intracellular parasites protect themselves against reactive oxygen intermediates by producing detoxifying enzymes of respiratory burst products. The ability of *Leishmania* sp. and *Toxoplasma gondii* to withstand reactive oxygen intermediates correlates with the amount of detoxifying enzymes (e.g. catalase, glutathione peroxidase, super oxide dismutase) found in these protozoans (Murray and Cohn, 1979; Murray et al., 1980a; Murray and Cohn, 1980; Murray, 1981). A similar correlation between reactive oxygen toxicity and detoxifying enzymes can also be seen in the bacterial pathogen, *Legionella pneumophila* (Locksley et al., 1982a).

Pathogens may also utilize receptors which do not induce respiratory burst responses. For example, *T. gondii* circumvents the respiratory burst response in resident peritoneal macrophages, while other obligate intracellular pathogens of macrophages, *L. donovani* and *L. tropica*, induce respiratory burst responses in this same population of cells (Murray and Cohn, 1979; Murray et al., 1980a, 1980b; Murray, 1981). Even stage-specific ligands, used for entry of different developmental stages of a given pathogen, may have the ability to circumvent the respiratory burst of macrophages. *L. donovani* amastigotes (tissue stage in the definitive mammalian host) are less effective in inducing the respiratory burst response of macrophages when compared to the promastigotes (insect developmental stage), thus favoring amastigote survival in the intracellular environment (Channon et al., 1984; Channon and Blackwell, 1985). The developmental stages of *Leishmania* parasites use both shared and unique receptors for gaining entry into macrophages (Guy and Belosevic, 1991; Guy and Belosevic, 1993; Guy and Belosevic, 1995). The ability of *L. major* to survive within the intracellular environment is thought to be a result of its ability to enter macrophages using complement receptors (Mosser and Edelson, 1987; Da Silva et al., 1989; Guy and Belosevic, 1993); receptors which do not significantly alter the respiratory burst response of the host macrophage (Wright and Silverstein, 1983; Yamamoto and Johnston, 1984).

Respiratory burst oxidase activity can also be altered by secreted or surface bound molecules of select pathogens. For example, *Yersinia enterocolitica* avoids killing by the respiratory burst by producing unique plasmid-encoded proteins which prevent phagocytosis (China et al., 1994) and cytoskeletal reorganization (Hartland et al., 1994). The ability of the YopD protein from *Y. enterocolitica* to affect cytoskeletal reorganization has led to the speculation that its presence may impair the assembly of the respiratory burst oxidase at the plasma membrane (Hartland et al., 1994). Similarly, *Francisella tularensis* produces a soluble acid phosphatase that suppresses respiratory burst activity of phagocytes (Reilly et al., 1996).

Nitric Oxide

Results from numerous studies indicated that the respiratory burst was not the only cytotoxic effector mechanism of activated macrophages, since phagocytes obtained from CGD patients often retained both microbicidal and microbistatic activity (Murray et al., 1985; Rothermel et al., 1986; Scott et al., 1985). Moreover, the addition of scavengers of reactive oxygen did not abrogate the microbicidal activity of macrophages to certain pathogens *in vitro* (Murray et al., 1985; Scott et al., 1985; Catterall et al., 1986; Rothermel et al., 1986).

In the early 1980's, it was shown that nitrogen oxides were a common by-product of metabolism, and that the treatment of rats with endotoxin resulted in increased nitrate production (Green et al., 1981 a,b; Wagner et al., 1983). Stuehr and Marletta (1985) demonstrated that supernatants of murine macrophage cultures treated *in vitro* with bacterial lipopolysaccharide (LPS) contained both nitrate and nitrite. Subsequent studies showed that the production of these nitric oxide (NO) by-products by macrophages correlated with an increased cytotoxicity against tumors (Hibbs et al., 1987 a,b; Iyengar

et al., 1987; Hibbs et al., 1988; Stuehr and Nathan, 1989) and pathogens (see Table 1. 3).

The enzyme responsible for production of NO is nitric oxide synthase (NOS), of which both inducible (iNOS) and constitutive (cNOS) isoforms have been identified and cloned from several mammalian species (Stuehr and Griffith, 1993). The isoform found in macrophages is of the inducible form (Yui et al., 1991; Xie et al., 1992), and requires the presence of Ca^{2+} and calmodulin for activity (Xie and Nathan, 1994). iNOS has a high affinity for calmodulin at the low intracellular Ca^{2+} concentrations found in the cytoplasm, and thus does not depend on transient Ca^{2+} influxes for activity, as is the case for cNOS (Cho et al., 1992; Xie and Nathan, 1994). Moreover, expression of functional recombinant iNOS in *E. coli* is dependent upon co-expression with calmodulin (Wu et al., 1996). Macrophage-derived iNOS also requires tetrahydrobiopterin, flavin adenine dinucleotide and flavin mononucleotide for activity (Stuehr, et al., 1990; Stuehr et al., 1991; Baek et al., 1993).

Inducible NOS exists as a dimer in murine and rat macrophages, each monomer being 130-150 kD (Yui et al., 1991; Xie et al., 1992). Guanidino nitrogen of L–arginine act as nitrogenous donors for the enzymatic reaction (Fig. 1.4, Hibbs et al., 1987a; Iyengar et al., 1987), and analogs of this compound, such as N^G-monomethyl-L–arginine (N^GMMLA), are potent inhibitors of this enzyme (Nathan, 1992). Studies addressing the subcellular localization of iNOS have demonstrated that enzymatic activity resides in the cytoplasm (Stuehr et al., 1989). Macrophage activation also induces increase in arginine transporter activity, a prerequisite for inducible nitric oxide production in macrophages (Shibazaki et al., 1996).

Inducible NO cytotoxicity is an antimicrobial mechanism primarily attributed to activated macrophages, although other cells such as fibroblasts (Wang et al., 1996), hepatocytes (Nussler and Billiar, 1993), eosinophils (Zanardo et al., 1997; del Pozo et al., 1997), neutrophils (Evans et al., 1996),

endothelial cells (Feder and Laskin, 1994), and epithelial cells (DeVera et al., 1996; Igietseme, 1996) can be stimulated to produce NO.

NO responses by murine macrophages show an initial lag phase (6-12 hr, Stuehr and Marletta, 1987), with maximal NO production occurring within the first 24 hr after induction (Stuehr and Marletta, 1987). The most common assay for determination of NO response is the Griess reaction (Green et al., 1982) which measures nitrite (a by-product of nitric oxide formation). Nitrite accumulates in activated macrophage culture supernatants for up to 96 hr, indicating prolonged action of the enzyme (Stuehr and Marletta, 1987). Free radical NO, the immediate reaction product produced by iNOS, is most likely the cytotoxic effector molecule, although other by-products, such as nitrite, nitrate, and nitrosamines may also be toxic to pathogens (Iyengar et al., 1987; Hibbs et al., 1988; Stuehr and Nathan, 1989; Nathan, 1992). Although the respiratory burst has no apparent role in the production of NO (Iyengar et al., 1987), superoxide anion, produced from the respiratory burst response, can react with nitric oxide to form peroxynitrite (ONOO-), a reactive intermediate known to have potent microbicidal activity (Ischiropolous et al., 1990; Denicola et al., 1993).

Nitric oxide has an extremely high affinity for iron (Traylor and Sharma, 1992) and exhibits its cytotoxic action by complexing with iron-containing enzymes. Both heme and non-heme enzymes are susceptible to NO (Nathan, 1992; Stamler, 1994). For non-heme proteins, nitric oxide actively attacks iron-sulfur clusters, complexing with sulfur to mediate the release of iron and iron-nitrosyl complexes (Lancaster et al., 1990; Drapier et al., 1991). Enzymes susceptible to NO include: the oxidoreductases of the mitochondrial electron transport chain, aconitase, protein kinase C, ferritin, indoleamine 2,3,- dioxygenase, and ribonucleotide reductase (Drapier and Hibbs, 1986; Hibbs et al., 1988; Stuehr and Nathan, 1989; Reif and Simmons, 1990; Kwon et al., 1991; Gopalakrishna et al., 1993; Thomas et al., 1994). These enzymes are involved in oxidative phosphorylation, mitochondrial

respiration, intracellular signaling, iron storage, tryptophan degradation and DNA replication, respectively. In addition, iNOS activity is suppressed by high concentrations of NO, indicating that NO may function as a feedback inhibitor of its own production (Assreuy et al., 1993; Griscavage et al., 1993; Weisz et al., 1996).

Most of the work regarding the antimicrobial activities of NO of macrophages has been done using mouse macrophages. The production and regulation of NO by human macrophages is controversial (Denis, 1994). Some authors have speculated that NO may not be an antimicrobial mechanism of human macrophages, based on the fact that the cytokines involved in induction of NO production in murine macrophages do not induce human macrophages to produce NO (Murray and Teitelbaum, 1992; Schneemann et al, 1993). However, several reports have demonstrated NO production by human macrophages (Denis, 1991b; Munoz-Fernandez, 1992b, Bose and Farnia, 1995; Nicholson et al., 1996). Interestingly, a similar scenario is seen when comparing macrophages obtained from two closely related ruminants; cattle and goats. Monocyte-derived macrophages obtained from cattle produce copious amounts of nitric oxide when stimulated with cytokines and LPS, whereas monocyte-derived macrophages from goats fail to produce NO when stimulated under the same conditions (Adler et al., 1996). Clearly, the regulatory pathways inducing NO production in mammalian macrophages may vary between species.

Iron Deprivation

Commonly associated with infection and/or neoplasia is reduced plasma iron content, a condition known as "anemia of infection and chronic disorder" (Lee, 1983). This condition was originally thought to be a pathological state induced by infection, but is now known to be a

physiological response to infection. The desired effect of iron deprivation is to decrease circulating iron, thereby preventing or limiting access of this critical metabolite to pathogens (Table 1.4) (Weinberg, 1984).

Information regarding the physiological role of iron in immunity to infectious diseases has been covered in two excellent reviews by Weinberg (1984) and Kent et al. (1994). Experimental evidence supporting iron deprivation as an antimicrobial mechanism can be seen by the following observations (Weinberg, 1984): 1) hosts mobilize iron-binding proteins at sites of infection; 2) hosts recruit iron withholding mechanisms in response to microbial infection; 3) increased iron withholding decreases incidence and intensity of infection; and 4) pathogenic microorganisms attempt to acquire iron from host tissues and fluids.

The total amount of iron within macrophages represents a significant proportion of the iron content in a vertebrate host. Macrophages accumulate iron due to phagocytosis of expired erythrocytes (Jacobs, 1977), and subsequently play a critical role in the regulation of iron metabolism during host defense and homeostasis.

Iron is transported systemically by serum transferrin, and enters cells through receptor-mediated endocytosis. Transferrin dissociates from its receptor within the acidified endolysosome, and released iron is transported into the cytoplasm via a transporter system (Nunez, et al., 1990). Internalized iron accumulates in labile iron pools and is compounded to low molecular weight proteins (Jacobs, 1977). This intracellular pool acts as a readily available source of iron for metabolic processes or for use by pathogens (Jacobs, 1977; Byrd and Horwitz, 1991; Weiss et al., 1993). Excess iron in the cell is transferred to ferritin for storage (Jacobs, 1977). Ferritin is one of many iron containing proteins that is susceptible to inhibition by NO (Reif and Simmons, 1990). Moreover, expression of ferritin is downregulated in activated macrophages, possibly through the effects of nitric oxide on iron response factors. Iron response factors are NO-sensitive enzymes whose

function is to regulate the status of iron in the cell (Byrd and Horwitz, 1993; Drapier et al., 1993; Weiss et al., 1993). Thus, iron withholding by macrophages is intricately complexed to production of NO.

Lactoferrin, another iron-binding protein primarily found within the granules of neutrophils, has an extremely high affinity for iron at low pH (Masson et al., 1969). Lactoferrin participates in killing of pathogens by binding iron at localized sites of infection (Weinberg, 1984; Byrd and Horwitz, 1991), and catalyzing the formation of hydroxyl radicals from superoxide anion produced by the respiratory burst (Ambrusso et al., 1981). When released by neutrophils, lactoferrin is scavenged by activated macrophages, augmenting their antimicrobial response (Byrd and Horwitz, 1991).

Activated murine macrophages also increase production of transferrin (Djeha et al., 1995), an iron binding protein which has two primary roles in modulating the immune response against infectious organisms. Intracellular transferrin binds free iron, limiting the availability of intracellular iron for organisms like *Legionella pneumophilia* , which cannot use iron complexed to transferrin (Byrd and Horwitz, 1991). Macrophage-derived transferrin is also an essential element for lymphocyte proliferation (Djeha et al., 1993; Djeha et al., 1995). In addition, activated macrophages down-regulate transferrin receptor expression, thereby limiting the amount of iron entering the cell (Hamilton et al., 1984a; Byrd and Horwitz, 1989, 1991, 1993).

Infectious organisms require iron for growth and survival, and their growth, in most cases, is enhanced when excess iron is present (Kent et al., 1994). As such, pathogens have evolved various mechanisms enabling them to obtain and sequester iron from their host. These mechanisms include: 1) the ability to synthesize protein receptors for iron transporting proteins such as transferrin and lactoferrin; 2) the production of siderophores, voracious iron chelating agents and receptors for these siderophores; and 3) the

synthesis of cytolytic compounds that lyse red blood cells, releasing a readily available source of iron in the form of hemoglobin (Weinberg, 1984).

Tryptophan Degradation

Tryptophan is an essential amino acid required by vertebrates, and is the least available amino acid for metabolism (Ozaki et al., 1987). Plants and select microorganisms can synthesis tryptophan *de novo* . Indoleamine 2,3-dioxygenase (IDO) is an inducible enzyme produced by macrophages that is involved in the catabolism of tryptophan (Taylor and Feng, 1991). This recently cloned enzyme is a 42 kD protein that uses superoxide anion to oxidize the pyrrole ring of tryptophan (Hayaishi et al., 1977; Shimazu et al., 1978; Dai and Gupta, 1990; Tone et al., 1990; Taylor and Feng, 1991; Kadoya et al., 1992). Induction of IDO within macrophages deprives intracellular pathogens of available tryptophan. Likewise, catabolic products of tryptophan degradation, such as hydroxyanthranilic acid, are also toxic to microbes (Taylor and Feng, 1991). The catabolic pathway involved in tryptophan degradation is illustrated in Figure 1.4.

Tryptophan degradation as an antimicrobial response was first suggested by Pfefferkorn (1984), who demonstrated that *T. gondii* required tryptophan for intracellular growth and survival in human fibroblasts. Inhibition of parasite growth was reversed by the addition of exogenous tryptophan to infected fibroblast cultures. The effect was not a result of decreased uptake of tryptophan, but rather a result of its degradation. Subsequent work by Byrne et al. (1986), demonstrated the importance of tryptophan degradation in inhibiting the growth of *Chlamydia psittaci* in epithelial cells. The importance of this antimicrobial mechanism in regulating the growth of these parasites in macrophages has since been demonstrated

(Byrne et al., 1989; Schmitz et al., 1989; Carlin et al., 1989; Paguirigan et al., 1994; Carlin et al., 1995).

Induction of Mammalian Macrophage Antimicrobial Mechanisms by Cytokines

Macrophages, in general, require two signals before they become fully activated or cytotoxic (Meltzer et al., 1987; Crawford et al., 1994). These signals include : 1) a "priming" signal that induces macrophages to enter a state during which cytotoxic responses may be upregulated or modified (but not initiated), or which cause the macrophage to become receptive for a specific trigger signal (Crawford et al., 1994); and 2) a "trigger" signal in which the macrophage will execute an appropriate cytotoxic effector response (Crawford et al., 1994). There is evidence that cytokines can act as either the priming or triggering signals, or both (Crawford et al., 1994). Microbial molecules (e.g. bacterial lipopolysaccharide) recognized by the macrophage usually act as triggering signals for macrophage cytotoxic effector responses (Crawford et al., 1994). Although the focus of this portion of the review deals primarily with how cytokines induce particular cytotoxic mechanisms in macrophages, it is also important to highlight the indispensable role of pathogen-derived molecules, in particular bacterial lipopolysaccharide (LPS), which in conjunction with cytokines activate macrophages.

Induction of the Respiratory Burst by Cytokines

Macrophages can be primed for enhanced respiratory burst responses when stimulated with select cytokines. Priming does not activate the

respiratory burst oxidase, but rather upregulates production of specific subunits of the respiratory burst oxidase. Thus, cytokine-primed macrophages generate more reactive oxygen intermediates upon phagocytosis of a pathogen, compared to unstimulated macrophages.

This priming effect of macrophage respiratory burst responses was first demonstrated using supernatants obtained from mitogen-stimulated lymphocyte cultures (Murray et al, 1980 a,b; Murray, 1981; Nagakawara et al., 1982; Nathan et al., 1983). Treatment of macrophages with lymphocyte supernatants induced priming of macrophage respiratory burst responses after 2-4 days of cultivation (Nagakawara et al., 1982). Direct evidence for a specific molecule that primed macrophage respiratory burst responses was published by Nathan et al. (1983), who showed that the lymphokine, IFN-γ, was the molecule primarily responsible for the macrophage priming effect of these supernatants.

IFN-γ induces both an increased affinity of the respiratory burst oxidase for NADPH (Sasada et al., 1983; Tsunawaki et al., 1984; Cassatella et al., 1985), as well as increased rates of gene transcription and message expression for the gp91phox protein (Newburger et al., 1988; Cassatella et al., 1990). Peak gp91phox mRNA transcripts occur 24-48 hr after stimulation with IFN-γ and gradually decline by 72 hr (Newburger et al., 1988). Peak mRNA expression corresponds roughly to the length of time required for IFN-γ to prime PMA-induced respiratory burst responses. Although the mechanism by which IFN-γ upregulates and induces increased affinity of the oxidase for NADPH is not known, a possible mechanism would be through the regulation of affinity of the multiple phosphorylation pathways. Interestingly, IFN-γ does not affect p47phox transcription, indicating that p47phox may not limit the overall activity of the oxidase (Newburger et al., 1988; Cassatella et al., 1990). The requirement for macrophages to be incubated for at least 48 hr with IFN-γ for priming of the respiratory burst response, is in contrast to the induction of the respiratory burst response in

neutrophils. Neutrophils are rapidly primed by IFN-γ, within a few hours of stimulation, which results in upregulation of protein expression and not increased affinity of the oxidase for NADPH (Cassatella et al., 1988; Newburger et al., 1988). These findings indicate that although the machinery required for the respiratory burst activity may be similar among phagocytes, the mechanisms that regulate their respective induction pathways differ.

Tumor necrosis factor alpha (TNF-α) is another potent stimulator of macrophage antimicrobial responses. TNF-α acts as an autocrine stimulus for enhancement of the respiratory burst function in macrophages (Ding et al., 1988; Deng et al., 1989), increasing expression of both p47phox and p67phox transcripts in macrophages (Green et al., 1994). Reactive oxygen intermediates produced during the respiratory burst response, in turn, augment production of TNF-α by macrophages (Chaudhri et al., 1989).

IL-3 can also prime the respiratory burst response of human monocytes (Ho et al., 1992; Laursen et al., 1994). The priming of monocytes by IL-3 can be partially explained by the increased production of TNF-α by stimulated monocytes (Cannistra et al., 1988; Ho et al., 1992).

IL-4 primes the respiratory burst response of murine macrophages, and co-stimulation with TNF-α results in an enhanced priming of respiratory burst response, greater than that produced by either factor alone (Phillips et al., 1990). Interestingly, IFN-γ antagonizes IL-4 priming of respiratory burst of murine macrophages (Phillips et al., 1990). In addition, the priming of macrophages for respiratory burst by IL-4 may be species specific since the same effect cannot be demonstrated in human macrophages (Phillips et al., 1992).

Other cytokines which prime the macrophage respiratory burst response are macrophage colony stimulating factor (M-CSF, Wing et al., 1985) and granulocyte/macrophage colony stimulating factor (GM-CSF, Reed et al., 1987). Interestingly, GM-CSF induces a rapid priming of the macrophage respiratory burst response. Mouse macrophages are maximally primed for

respiratory burst activity after only 24 hrs of stimulation with GM-CSF (Reed et al., 1987). However, the amount of reactive oxygen generated by GM-CSF-stimulated macrophages is considerably less than in macrophages primed with IFN-γ (Reed et al., 1987).

Bacterial lipopolysaccharides (LPS) can also prime macrophage respiratory burst responses (Pabst et al., 1980; Pabst et al., 1982; Phillips and Hamilton, 1989; Green et al., 1994). However, some studies have reported an inhibitory effect of LPS on the generation of reactive oxygen intermediates (Johnston et al., 1985; Johnson and Sung, 1987; Ding and Nathan, 1987; Rellstab et al., 1989; Bogdan et al., 1993), and that LPS acts as an antagonist of the priming effects induced by IFN-γ (Ding and Nathan, 1987). For example, pretreatment of murine resident peritoneal macrophages with LPS for 1-4 hr, before IFN-γ, treatment abrogates the priming of macrophages by IFN-γ measured after 48 hr (Ding and Nathan, 1987).

The ability of cytokines to prime the respiratory burst response of macrophages also depends on the sub-population of macrophage studied. Monocytes are thought to exist in a semi-primed state *in vivo*, since freshly isolated monocytes triggered with PMA exhibit a vigorous respiratory burst response (Pabst et al., 1982; Cassatella et al., 1985). In contrast, monocytes cultured *in vitro* (monocyte-derived macrophages) have reduced respiratory burst activity that correlates with the length of *in vitro* cultivation (Pabst et al., 1982; Cassatella et al., 1985). The *in vitro* stimulation of monocytes with IFN-γ augments pathogen-triggered respiratory burst responses, but has no effect on priming of the respiratory burst activity of other macrophage populations , such as alveolar macrophages, when triggered with the same stimuli (Kemmerich et al., 1987). The respiratory burst of bone marrow-derived macrophages can be triggered with yeast zymosan but not PMA, while resident peritoneal macrophages show basal respiratory burst activity when triggered with PMA (Phillips and Hamilton, 1989). Bone marrow-derived macrophages become responsive to PMA as a trigger signal when

cultured in the presence of GM-CSF, TNF-α, IFN-γ or LPS (Phillips and Hamilton, 1989). Liver macrophages (Kupffer cells) lack a basal respiratory burst response, and cannot be primed for respiratory burst activity by stimulation with IFN-γ (Lepay et al., 1985). The unresponsive state of Kupffer cells to both LPS and cytokines is important, because the liver is the site where nutrients, and molecules such as LPS, end up after uptake across the small bowel (Lepay et al., 1985). This variability in respiratory burst responses between different sub-populations of macrophages suggests, that the microenvironment in which the macrophage is found is critical in controlling respiratory burst regulatory signals perceived by that macrophage.

Induction of Nitric Oxide by Cytokines

Murine macrophages stimulated with LPS or IFN-γ produce small amounts of nitric oxide (NO) in comparison to the synergistic production observed after treatment with both factors (Stuehr and Marletta, 1987; Iyengar et al., 1987). The NO response of murine macrophages following LPS or IFN-γ stimulation is dose dependent, as is the synergy in nitric oxide response induced after treatment of macrophages with both factors (Stuehr and Marletta, 1987). Stimulation with either factor results in the upregulation of iNOS gene transcription. The synergism between LPS and IFN-γ appears to be a result of increased transcription rates of the iNOS gene (Lorsbach et al., 1993), and increased stability of iNOS mRNA transcripts (Weisz et al., 1994).

The mechanism of LPS induction of NO production and its synergy with IFN-γ are linked to the induction of another cytokine produced by macrophages, TNF-α. Treatment of macrophages with LPS induces TNF-α production, which is enhanced in the presence of IFN-γ (Farrar and Schreiber,

1993). TNF-α synergizes with IFN-γ for the induction of NO response in murine macrophages, but does not induce NO production by itself (Drapier et al., 1988; Ding et al., 1988; Liew et al., 1990 a,b; Munoz-Fernandez et al., 1992 a,b). The addition of monoclonal antibodies against TNF-α to cultures stimulated with LPS and IFN-γ drastically reduces NO production by macrophages (Drapier et al., 1988). The addition of anti-TNF-α antibodies to cultures of IFN-γ-treated murine macrophages infected with *L. major* completely abrogates the production of NO by these macrophages (Green et al., 1991; Nacy et al., 1991).

On the other hand, data exist to suggest that this TNF-α-dependent induction of NO production is not the only mechanism by which LPS exerts its indirect NO-inducing potential. Addition of anti-TNF-α antibodies to macrophage cultures treated with LPS results in decreased NO response but not complete suppression of NO production (Drapier et al., 1988). Pre-treatment of macrophages with low concentrations of LPS before the addition of IFN-γ to macrophage cultures results in a dose dependent inhibition of NO production (Bogdan et al., 1993). Interestingly, very small amounts of LPS downregulate NO production, while causing increased TNF-α production (Zhang and Morrison, 1993). Moreover, the addition of tyrosine kinase inhibitors to LPS /IFN-γ co-stimulated murine macrophages inhibits iNOS but does not affect production of TNF-α (Orlicek et al., 1996)

Type I interferons, IFN-α and IFN-β, are also important autocrine modulators of LPS-induced NO production by inflammatory murine peritoneal macrophages (Ding et al., 1988; Zhang et al., 1994). Monoclonal antibodies to IFN-β abrogate LPS-induced NO production (Zhang et al., 1994). Interestingly, the addition of exogenous IFN-β to the macrophage cultures results in very little NO production, and the addition of TNF-α to cultures does not enhance the NO response (Zhang et al., 1994). These data suggest that, although IFN-β may be necessary for LPS-induced NO production, co-stimulatory factors (other than TNF-α) are required for the induction of NO

response by LPS (Zhang et al. 1994). Although both IFN-α and IFN-β induce NO production in macrophages, this response is considerably lower when compared to that induced by IFN-γ (Ding et al., 1988). The mechanisms by which IFN-α and IFN-β induce macrophage NO response are not known, but may be related to their ability to induce tryptophan degradation (Varesio et al., 1990; Melillo et al., 1993; 1996 a,b; 1997 a,b).

Other cytokines are also thought to be important for the induction of nitric oxide in macrophages. For example, IL-2 is an important macrophage-activating cytokine that in combination with IFN-γ induces macrophage cytotoxicity (Belosevic et al., 1988; Belosevic and Nacy, 1990; Belosevic et al; 1990; Nelson et al., 1992). Treatment of murine macrophages with IL-2 in conjunction with IFN-γ results in a significant macrophage NO response (Cox et al., 1992; Deng et al., 1993). Although treatment of murine macrophages with IL-2 and IFN-γ also induces TNF-α production (Strieter et al., 1989; Economou et al., 1989), IFN-γ-treated murine macrophages subsequently treated with saturating quantities of TNF-α and IL-2 exhibit an even higher NO response (Deng et al., 1993).

To date, most of the work regarding the regulation of NO production by cytokines has been done using murine macrophages. The production and regulation of NO by human macrophages is controversial. Some authors have speculated that NO may not be an antimicrobial mechanism of human macrophages, based on evidence that cytokines that induce mouse macrophages to produce NO *in vitro* do not appear to induce NO production in human monocytes (Murray and Teitelbaum, 1992; Schneemann et al., 1993). However, recent experiments have demonstrated the *in vitro* production of NO by human macrophages after cytokine treatment and intracellular infection (Denis, 1991a; Munoz-Fernandez, 1992b; Denis, 1994; Nicholson et al., 1996). In these studies, human macrophages were first exposed to pathogens (*Mycobacterium avium* , *M. tuberculosis* , or *Trypanosoma cruzi*), and then treated with TNF-α, or IFN-γ and TNF-α. This combined

stimulus of intracellular infection and cytokines resulted in the accumulation of significant amounts of nitrite in culture supernatants. These findings indicate that the production of NO by human macrophages may be regulated differently than that of murine macrophages (Denis, 1994). The precise cytokine and pathogen stimuli may also be important, since some studies were unable to show NO production by human macrophages after treatment with IFN-γ and/or intracellular infection (Murray and Teitelbaum, 1992). As stated previously, similar results have been reported for bovine and caprine macrophages, where treatment of bovine macrophages with IFN-γ and LPS results in NO production, but stimulation of goat macrophages with these same agents fails to induce NO production (Adler et al., 1996).

Induction of Iron Deprivation by Cytokines

Relatively little is known regarding the mechanisms of cytokine induced regulation of iron metabolism in macrophages. It is perhaps not surprising that IFN-γ is a critical cytokine that regulates iron metabolism in activated macrophages. IFN-γ induces the production of transferrin by murine but not human macrophages (Djeha et al., 1995). In addition, IL-1, IL-6 and TNF-α can also induce transferrin synthesis by murine macrophages, albeit to a lesser extent than IFN-γ (Djeha, 1995). Transferrin produced by murine macrophages contains bound iron and is a potent co-stimulator of T-lymphocyte proliferation (Djeha et al., 1993). The ability of murine macrophages to produce iron-containing transferrin is thought to be a critical mechanism by which lymphocytes can still undergo extensive proliferation while the body limits the amount of iron available systemically (Djeha et al., 1995). While human macrophages can not produce transferrin in response to IFN-γ activation, human T-cells produce transferrin when appropriately stimulated (Djeha et al., 1995).

IFN-γ also downregulates the surface expression of transferrin receptors and intracellular ferritin in human and murine macrophages (Hamilton et al., 1984b; Byrd and Horwitz, 1989; Byrd and Horwitz, 1993), resulting in a decrease in intracellular iron content and the uptake of circulating extracellular iron. Fahmy and Young (1993) showed that TNF-α and IL-1 can also downregulate transferrin receptor expression in a human monocyte cell line, while IFN-γ had no effect on the expression of transferrin receptors in this system.

Induction of Tryptophan Degradation by Cytokines

Indoleamine 2,3-dioxygenase is an interferon-inducible protein (Taylor and Feng, 1991). IFN-γ is the most potent stimulator of IDO activity and tryptophan degradation (Ozaki et al., 1987; Carlin et al., 1989; Werner-Felmayer et al., 1989; Werner-Felmayer et al., 1990; Hissong et al., 1995). Induction of IDO by IFN-γ can be augmented by co-stimulating macrophages with TNF–α (Werner-Felmayer et al., 1990). IFN-α and IFN-β also induce IDO and tryptophan degradation, albeit to a much lesser extent than IFN–γ (Ozaki et al., 1987; Carlin et al., 1989; Werner-Felmayer et al., 1989; Werner-Felmayer et al., 1990; Hissong et al., 1995). The addition of LPS to human monocytes stimulated with IFN-α and IFN-β enhances tryptophan degradation, comparable to that induced by IFN-γ only (Schmitz et al., 1989; Werner-Felmayer et al., 1990). The mechanism by which LPS enhances IFN-β-induced degradation of tryptophan may involve an autocrine production of TNF-α by macrophages. Treatment of monocytes with TNF-α alone induces only minor amounts of IDO activity, whereas co-stimulation with IFN-α and IFN-β enhances tryptophan degradation (Werner-Felmayer et al., 1990). In addition, IL-1α can enhance both IFN-β and IFN-γ induction of IDO (Carlin and Weller, 1995; Hissong et al., 1995).

Evidence also suggests that tryptophan degradation can itself enhance other antimicrobial functions of activated macrophages. Picolinic acid, a metabolite of tryptophan degradation, augments NO responses in IFN-γ–treated murine macrophages, and synergizes with IFN-γ for induction of macrophage tumoricidal activity (Varesio et al., 1990; Melillo et al., 1993; 1996 a,b; 1997 a,b). It appears that tryptophan degradation is linked to macrophage NO production, due to the known effects of picolinic acid induction on NO production, and the susceptibility of IDO to NO (Thomas et al., 1994).

Antimicrobial Mechanisms of Teleost Macrophages and Their Induction by Cytokines

The study of comparative macrophage biology is a newly emerging field. Consequently, little is known about macrophage antimicrobial mechanisms, or their regulation, in lower vertebrates such as fish. This portion of the literature review is a synopsis of our current understanding of these mechanisms in teleosts. This section has three purposes: 1) to highlight literature prior to 1992, thereby providing the framework from which this thesis originated; 2) to review current developments in this area; and 3) to outline the contributions that my research has made to our current understandings of the molecular mechanisms regulating macrophage-mediated immunity in fish.

Up until 1992, the only inducible macrophage antimicrobial response to be identified in fish was the respiratory burst. Only one study has attempted to characterize the biochemical nature of the enzymes involved in elicitation of the respiratory burst. Secombes et al. (1992), demonstrated the presence of a low potential b-type cytochrome that localized to the plasma

membrane of rainbow trout macrophages, a phenomenon similar to that observed in mammalian phagocytes.

Significantly more work has been done regarding the regulation of teleost macrophage respiratory burst response by cytokines. However, since purified or recombinant fish cytokines are not available, studies addressing the regulation of macrophage activation in teleosts have relied on crude cytokine preparations. These crude cytokine preparations are obtained from mitogen-stimulated kidney leukocytes which secrete macrophage activating factor(s) (MAF) into culture medium upon stimulation. The supernatants from these cultures are subsequently used to activate teleost macrophage antimicrobial mechanisms.

The respiratory burst of rainbow trout macrophages can be primed *in vitro* by stimulating resident kidney macrophages with culture supernatants obtained from mitogen-stimulated kidney leukocytes (Chung and Secombes, 1987; Graham and Secombes, 1988, 1990). Teleost macrophages require extensive cultivation periods (i.e. 48 hrs) with these supernatants for priming of their respiratory burst, an effect shared with their mammalian counterparts (Nagakawara et al., 1982). The MAF within these crude cytokine preparations was shown to be a secretory product of fish T-cells (Graham and Secombes, 1990a). Production of MAF was also shown to be induced through antigen-specific stimulation of lymphocyte cultures *in vitro* (Francis and Ellis, 1994; Marsden et al., 1994; Yin et al., 1997). In addition to the ability of crude MAF preparations to prime the respiratory burst, they also induce adherence and spreading of macrophages in culture (Secombes, 1987). Its functional activities, cellular source, and biochemical nature (Graham and Secombes, 1990b) suggest that the molecule in crude cytokine preparations responsible for MAF activity, may be the teleost equivalent of mammalian IFN-γ (Graham and Secombes, 1988, 1990 a,b).

Although the molecule(s) responsible for MAF activity has yet to be purified, there is evidence that a TNF-α-like molecule may be responsible for

at least some of the MAF activity seen in mitogen-stimulated leukocyte supernatants (Hardie et al., 1994; Jang et al., 1995b; Novoa, 1996; Tahir and Secombes, 1997; Campos-Perez et al., 1997). Human recombinant TNF-α synergizes with MAF to enhance priming of respiratory burst activity in rainbow trout macrophages (Hardie et al., 1994; Tahir and Secombes, 1997; Campos-Perez et al., 1997). Moreover, priming of the respiratory burst can be partially inhibited using anti-human TNF-α receptor 1 monoclonal antibodies, suggesting a certain degree of conservation in both the TNF molecule and its receptor between mammals and teleosts (Jang et al., 1995b).

Another molecule that appears to affect respiratory burst function in teleost macrophages is growth hormone. Recombinant rainbow trout growth hormone has been shown to prime sea bass (*Dicentrarchus labrax*) macrophage respiratory burst response after 24 hrs of stimulation (Calduch-Giner et al., 1997; Munoz et al., 1988).

In contrast to the findings described above, I have observed that goldfish macrophages stimulated with MAF supernatants undergo rapid priming of their respiratory burst (Neumann and Belosevic 1996; Chapters IV and V). These primed macrophages are subsequently "de-primed", such that by 24-48 hrs after stimulation their respiratory burst responses are similar to those of unstimulated macrophages (Neumann and Belosevic, 1996; Chapters 4 and 5). Respiratory burst de-primed macrophages are highly cytotoxic, in that they produce copious amounts of nitric oxide. Thus, it would appear that teleost macrophages mount sequential cytotoxic responses (Neumann and Belosevic, 1996; Chapters 4 and 5), a phenomenon not previously attributed to mammalian macrophages. The rapid priming and de-priming observed in goldfish macrophages may be a result of the concerted efforts of the multiple MAF activities present in crude cytokine preparations. My research has demonstrated that supernatants from mitogen-stimulated kidney leukocytes contain multiple MAFs: molecules that can either prime teleost macrophage respiratory burst responses, induce nitric oxide

production, or synergize for enhanced macrophage activation (Chapter 5). In addition, these crude cytokine supernatants also contain macrophage deactivating factors, molecules that can reduce or inhibit the activation state of macrophages. Moreover, different macrophage sub-populations, raised by *in vitro* cultivation (Neumann et al., 1998; Chapter III), show different kinetics of activation when stimulated with combinations of these MAFs (Chapter III).

The respiratory burst of fish macrophages can also be primed by bacterial LPS (Solem et al, 1995; Neumann et al., 1996; Campos-Perez, 1997), β-glucans from yeast cell walls (Robertsen et al., 1994; Brattgjerd et al., 1994; Dalmo and Saljelid, 1995; Jorgensen and Robertsen , 1995; Tahir and Secombes, 1997), or bacterial proteins (Francis and Ellis, 1994). Priming of the respiratory burst by these agents also requires extensive cultivation periods (i.e. $\geq$ 48 hrs). Macrophage respiratory burst activity can be primed *in vivo* by administering killed or attenuated bacterial pathogens (Chung and Secombes, 1987; Enane et al., 1993; Marsden et al., 1994; Yin et al., 1997).

Inducible nitric oxide production by fish macrophages has been a recent discovery. Although constitutive nitric oxide synthase had been demonstrated from the central nervous system of fish (Ostholm et al., 1994; Holmqvist et al., 1994) only one report prior to 1995 had demonstrated that fish possess an inducible form of this enzyme. Schoor and Plum (1994), demonstrated inducible nitric oxide production, using enzyme histochemical techniques, from kidney homogenates obtained from channel catfish (*Ictalurus punctatus*) infected with the bacterium *Edwardsiella ictaluri* . Our laboratory subsequently demonstrated, for the first time, that nitric oxide production could be induced in goldfish macrophages stimulated with bacterial LPS (Wang et al., 1995). My research subsequently demonstrated that nitric oxide could be synergistically induced in goldfish macrophages stimulated with LPS and MAF (Chapter 3, 4, 5; Neumann et al., 1995, Neumann and Belosevic, 1996; Neumann et al, 1998). Collaborations

between our laboratory and others resulted in the cloning of a partial nucleotide sequence of goldfish macrophage iNOS. This partial goldfish iNOS nucleotide sequence shares 70% homology with mammalian macrophage iNOS (Laing et al., 1996). More recent studies have also demonstrated nitric oxide production by macrophages of other species of teleosts (Sakai et al., 1997; Mulero and Meseguer, 1998).

Unlike mammals, where numerous studies have been conducted, relatively few studies have examined the cytotoxic capability of fish macrophages *in vitro*. Graham and Secombes (1988) demonstrated that rainbow trout macrophages stimulated with crude MAF preparations could inhibit growth of the bacterium *Aeromonas salmonicida*, etiological agent of furunculosis. Subsequent work by this group, and others, demonstrated that killing of select pathogens correlated with the production of reactive oxygen intermediates, and the addition of scavengers of reactive oxygen, such as catalase, abolished the ability of macrophages to restrict the growth of pathogens (Hardie et al., 1996; Yin et al, 1997; Campos-Perez et al., 1997). Most recently, Yin et al., (1997) demonstrated that MAF-activated catfish macrophages were bactericidal towards *Aeromonas hydrophila* , and that killing could be partially blocked using N^GMMLA, an inhibitor of nitric oxide production. Together, the data clearly suggest a role for both reactive oxygen and nitrogen intermediates in killing or restricting the growth of certain fish pathogens.

Currently, nothing is known as to the contributions that iron deprivation and tryptophan degradation play as inducible antimicrobial responses of fish macrophages.

Table 1.1. Cytokine-inducible antimicrobial mechanisms of vertebrate macrophages.

Generalized Antimicrobial Response	Mechanism	Molecule or enzyme responsible for activity
Production of cytotoxic molecules	• Formation of reactive oxygen intermediates	respiratory burst oxidase
	• Formation of reactive nitrogen intermediates	nitric oxide synthase
Nutrient deprivation	• Degradation of tryptophan	indoleamine 2,3-dioxygenase
	• Iron deprivation	nitric oxide

Table 1.2. Mammalian pathogens and their susceptibility to various reactive oxygen products produced during the respiratory burst.

Pathogen	Reactive oxygen product to which pathogen is susceptible	References
Legionella pneumophila	OH·	Jacobs et al. 1984. Locksley et al., 1982a.
Candida albicans	HOCl	Marodi et al., 1991.
Leishmania sp.	H_2O_2	Murray, 1981; Channon et al., 1985; Channon and Blackwell, 1985; Locksley et al., 1982b.
	OH·	Zarley et al., 1991.
Toxoplasma gondii	OH·	Murray and Cohn, 1979; Murray et al., 1985.
Entamoeba histolytica	H_2O_2	Denis and Chadee, 1989.
Trypanosoma cruzi	H_2O_2	Nathan et al.,1979.
	ONOO-	Denicola et al., 1993.

Table 1. 3. Mammalian pathogens susceptible to reactive nitrogen intermediates.

Pathogen	Reference
Listeria monocytogenes	Bermudez, 1993.
Mycobacterium sp.	Doi et al., 1993; Denis, 1991 a,b; Adams et al., 1991.
Legionella pneumophilia	Summersgill et al., 1992.
Francisella tularensis	Green et al., 1993.
Ehrlichia risticii	Park and Rikihisa, 1992.
Chlamydia trachomatis	Mayer et al., 1993.
Candida albicans	Cenci, et al., 1993.
Rickettsia conorii	Feng et al., 1994.
Cryptococcus neoformans	Brummer and Stevens, 1994; Granger et al., 1988.
Toxoplasma gondii	Bohne et al., 1994; Gazzinelli et al., 1993; Langermans et al., 1992.
Leishmania sp.	Liew et al., 1990 a,b; Green et al., 1990; Green et al., 1991; Severn et al., 1993; Assreuy et al., 1994.
Trypanosoma sp.	Gazzinelli et al., 1992; Denicola et al., 1993; Munoz-Fernandez et al., 1992 a,b; Sternberg et al., 1994; Vespa et al., 1994 .
Plasmodium yoeli	Nussler et al., 1991.
Entamoeba histolytica	Lin et al., 1994.
Schistosoma mansoni	Wynn, et al., 1994; James et al., 1989.

Table 1.4. Pathogens susceptible to macrophage-induced iron deprivation.

Pathogen	Reference
Legionella pneumophilia	Alford et al., 1991; Byrd and Horwitz, 1989; Byrd and Horwitz, 1991; Gebran et al., 1994.
Histoplasma capsulatum	Lane et al., 1991.
Ehrlichia risticii	Park and Rikihisa, 1992.

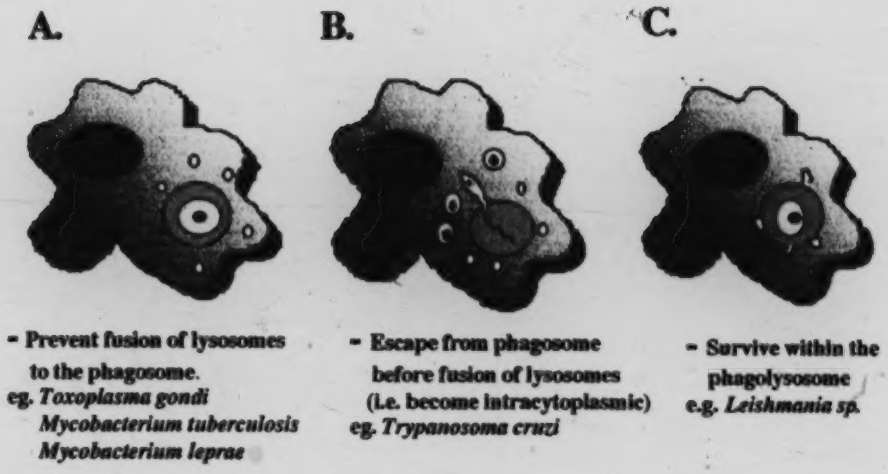

Figure 1.1. Intracellular survival of pathogens depends on their ability to evade lysosomal killing upon ingestion by phagocytes. Illustrated above are three strategies commonly used by parasites to ensure intracellular survival and replication within macrophages.

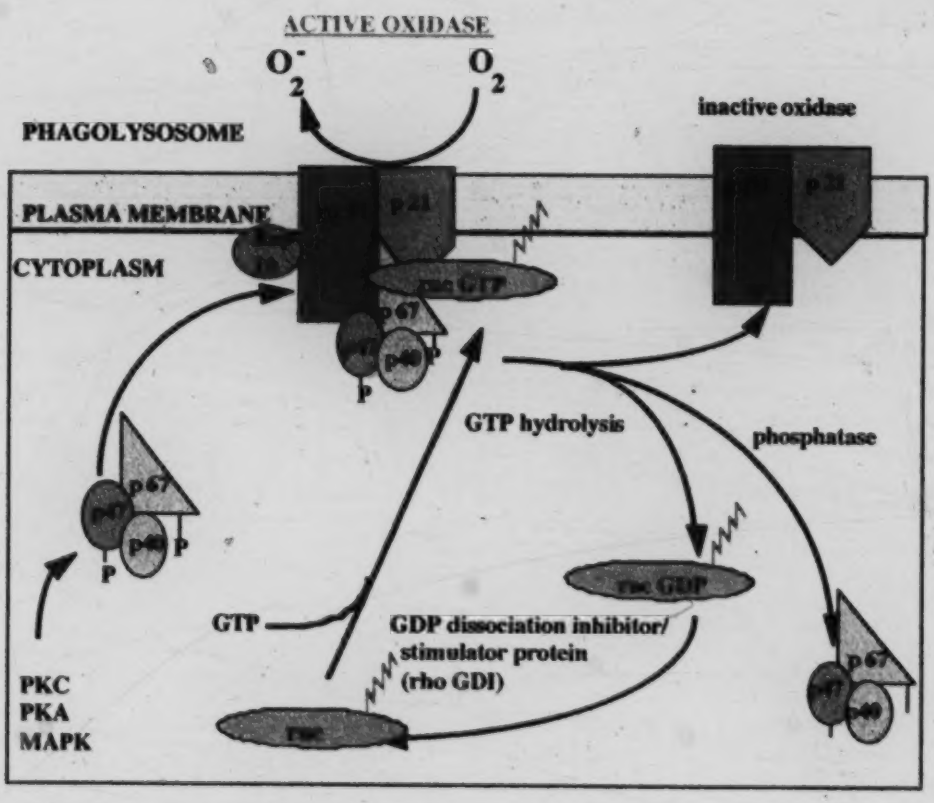

Figure 1.2. Assembly of the respiratory burst oxidase within the membrane of phagocytes. Phosphorylation of cytosolic components (p47 and p67) by protein kinase C (PKC), protein kinase A (PKA), or mitogen activated protein kinase (MAPK) initiates translocation of cytosolic components to the cell membrane. At the membrane, these cytosolic components interact with membrane bound components (gp 91 and p21), and in the presence of rac proteins, form the functional respiratory burst oxidase. Activity of this multicomponent enzyme is regulated by: 1) GDP dissociation inhibitor/stimulator proteins, such as rho GDI, that regulate dissociation of hydolyzed GDP to rac proteins, and 2) by phosphatases that regulate levels of phosphorylated cytosolic components.

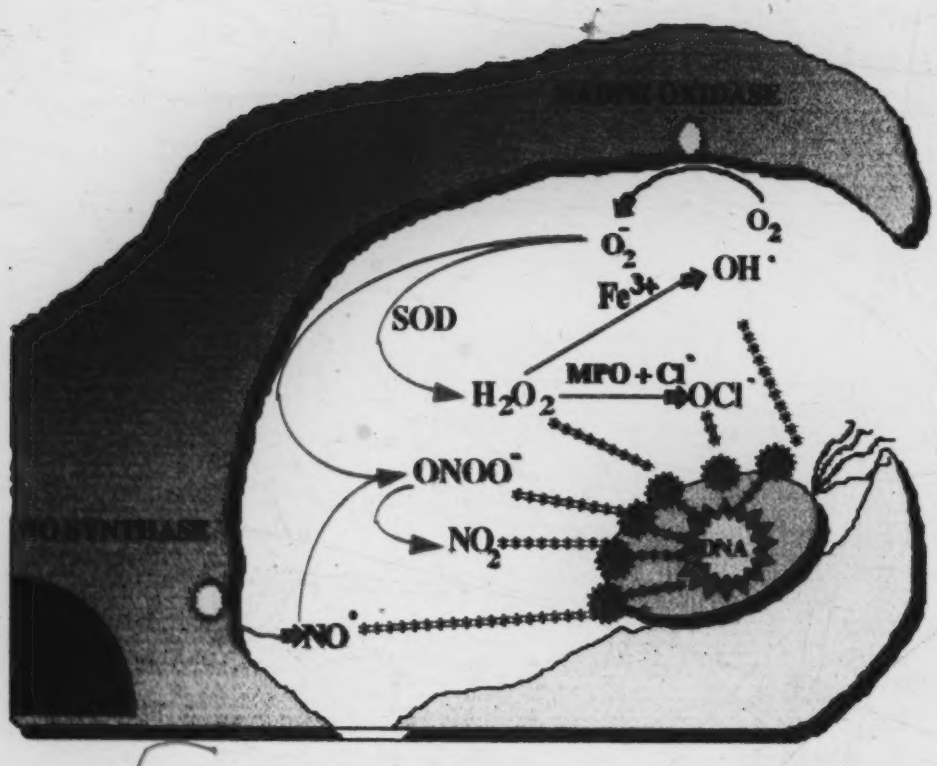

Figure 1.3. Reactive oxygen and nitrogen intermediates produced by activated macrophages. Superoxide (O_2^-) produced via the respiratory burst is dismutated to hydrogen peroxide (H_2O_2) in the presence of the enzyme superoxide dismutase (SOD). H_2O_2 can react with ferric iron (Fe^{3+}) to form hydroxyl radicals ($OH^\cdot$), or react with chloride ions (Cl^-) in the presence of the myleoperoxidase (MPO), to form hypochlorous acid (OCl^-). Superoxide can also react with nitric oxide ($NO^\cdot$) to form a highly reactive oxidant known as peroxynitrite (ONOO-). Peroxynitrite can spontaneously mutate to nitrite or nitrate (NO_2, NO_3). These toxic reactive oxygen and nitrogen intermediates cause extensive lipid peroxidation, protein hydrolysis/oxidation, as well as DNA fragmentation.

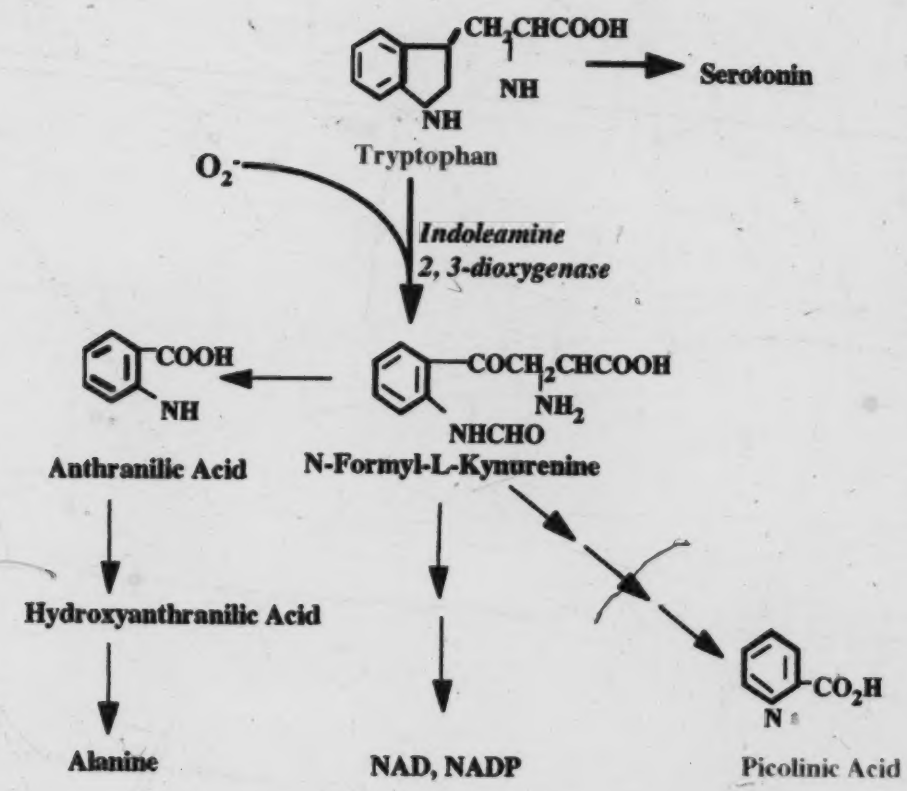

Figure 1.4. Immune-mediated degradation of tryptophan in activated macrophages.

CHAPTER II

GENERAL MATERIALS AND METHODS

Fish

Goldfish (*Carassius auratus*) 10 to 15 cm in length were purchased from either Ozark Fisheries Inc. (Southland, MI) or Grassy Forks Fisheries (Martinsville, IN) and maintained at the Aquatic Facility of the Department of Biological Sciences, University of Alberta. The fish were held at 20°C in a flow-through water system on a simulated natural photoperiod (Edmonton, Alberta) for the duration of the experiments. The fish were fed to satiation daily with trout pellets. They were acclimated to this environment for at least 3 weeks prior to use.

Culture medium and culture conditions

The culture medium used in all experiments was a modified form of that previously described for culturing a goldfish macrophage cell line (GMCL, Wang et al., 1995). The formulation for the medium is listed in Table 2.1. Modifications of the original medium provided a powerful buffering capacity for culturing goldfish macrophages and leukocytes in medium exposed to air. Complete medium contained 100 U penicillin, 100 µg/ml streptomycin, 100 µg/ml gentamicin, 10% newborn calf serum (Hyclone), and 5% goldfish or carp serum. All goldfish cell cultures were grown at 20°C, unless otherwise specified.

Fish serum

Fish serum was obtained by bleeding large goldfish or common carp (15-40 cm). The fish were anaesthetized using tricaine methanosulfonate (approximately 40 mg/l) and bled by caudal venipuncture. These fish were maintained solely for serum production and were bled every 8-10 weeks. Blood collected from these fish was allowed to clot at room temperature for approximately 1 hr and overnight at 4°C. The following morning blood samples were centrifuged at 1000 x *g* for 20 min and serum removed. Serum was heat inactivated at 56°C for 30 min, filter sterilized (0.22 μM), and stored at -20°C until used.

Reagents

The following is a list of the chemicals and reagents (and their respective suppliers) used in the experiments described in this thesis: 2,3-bis[2-methoxy-4-nitro-5-sulfo-phenyl]-2H-tetrazolium-5-carboxanalide (XTT, Sigma); calcium ionophore A23187 (Sigma); concanavalin A (ConA, Boehringer Mannheim); 2,3,-dimethoxy-5-methyl-1,4-benzoquinone (co-enzyme Q, Sigma); dimethyl sulfoxide (DMSO, Caledon); Dulbecco's Modified Eagle Medium (DMEM, Gibco); gentamicin (Gibco); heparin (Sigma); lipopolysaccharide (LPS) obtained from *Salmonella typhimurium* (Difco); N-naphthylethylene diamine (Sigma); nitroblue tetrazolium (NBT, Sigma); penicillin/streptomycin (Gibco); Percoll (Pharmacia); phorbol myristate acetate (PMA, Sigma); phosphoric acid (Anachemia); polyethylene glycol (PEG, Sigma); saponin (Sigma); sulfanilamide (Sigma); tricaine methanosulfonate (Syndel Laboratories); trypan blue (Gibco).

Mammalian cell lines

Two mammalian macrophage cell lines were used in select experiments for comparing macrophage responses between mammals and teleosts. J774A.1, a murine macrophage cell line, was maintained by serial passage in DMEM (Gibco) containing 10% fetal bovine serum. U937, a human monocyte-like cell line, was maintained by serial passage in RPMI 1640 (Gibco) containing 10% fetal bovine serum.

Isolation of goldfish kidney leukocytes

Leukocytes were isolated from the kidneys of goldfish using procedures described previously (Chung and Secombes, 1987; Graham and Secombes 1988, 1990 a,b). Fish were anesthetized with tricane methanosulfonate (approximately 40 mg/l), and the kidneys aseptically removed and placed in ice-cold medium. The kidneys were pressed through sterile stainless steel screens with medium containing antibiotics (100 µg/ml gentamicin, 100 U/ml penicillin, 100 µg/ml streptomycin) and heparin (50 U/ml). The cell suspension was layered on 51% Percoll and centrifuged at 400 x *g* for 25 minutes. Cells at the medium-51% Percoll interface were transferred into clean tubes and washed twice by centrifugation at 200 x *g* for 10 min at 4°C. The number of viable leukocytes was determined using a trypan blue exclusion assay and enumerated using a haemocytometer.

Production of MAF

Experiments were conducted to optimize conditions for generating MAF preparations from mitogen-stimulated kidney leukocytes. Several parameters were examined that could potentially influence MAF production. These included mitogen concentration, initial kidney leukocyte seeding density, temperature, presence or absence of serum, and duration of incubation.

Effect of mitogen concentration on MAF production

To assess the effects of mitogen concentration on MAF production, $2x10^6$ goldfish kidney leukocytes, in 1 ml of medium supplemented with serum and antibiotics, were placed in each well of a 24 well tissue culture plate. Cells were stimulated with 0, 2.5, 5, 10, 15 or 20 µg of Con A and/or 5, 10, or 20 ng of PMA. All cultures stimulated with PMA were also treated with 100 ng calcium ionophore A23187, since previous reports have shown that stimulation with both PMA and calcium ionophore results in synergistic cell proliferation and cytokine production in fish (Miller and Clem, 1988; Graham and Secombes, 1988). The leukocytes were incubated with mitogens for 6 hours at 20°C, and cell monolayers gently washed 3X with Hanks balanced salt solution (HBSS) to remove mitogens. Two ml of serum-free medium were then added to the remaining adherent cell layer. One ml of the cell supernatant was immediately removed and used as a control to ensure complete removal of mitogens. The leukocytes were incubated for an additional 72 hrs at 20°C. MAF supernatants from three individual fish were pooled for each treatment condition, filter-sterilized (0.22 µM filter, MilliPore), and stored at -20°C until assayed for MAF activity. MAF activity was assayed for its ability to induce nitric oxide production in goldfish macrophages (see below).

Effect of cell number on MAF production

Effect of cell seeding density on MAF production was determined by seeding 1, 2, 4, 8, or 10 x 10^6 cells/ml in 24 well culture plates and stimulating the cells with 10 µg/ml ConA, 10 ng/ml PMA, and 100 ng/ml calcium ionophore A23187 (determined to be the optimal concentrations of mitogens for MAF production). Cells were washed three times with HBSS and cultured for 72 hrs. Supernatants were removed, filter sterilized and assayed for MAF activity using the nitric oxide assay.

Effect of temperature, duration of cultivation, and presence of serum on MAF production

Four times 10^6 kidney leukocytes were placed in 1 ml of medium in 24 well culture plates and stimulated with 10 µg of Con A, 10 ng PMA and 100 ng calcium ionophore for 6 hrs. Cultures were subsequently washed three times with HBSS, and incubated in medium with or without serum for 0, 24, 48, 72 or 96 hrs at 10°C, 20°C or 30°C. Supernatants were removed, filter sterilized and assayed for MAF activity using the nitric oxide assay.

Batch production of MAF

Initial results demonstrated that optimal production of MAF occurred when 4x10^6 leukocytes/ml were stimulated with 10 µg/ml ConA, 10 ng/ml PMA, and 100 ng/ml calcium ionophore A23187. MAF was subsequently generated through the mitogenic stimulation of mixed kidney leukocyte populations using these conditions. MAF was raised in batch cultures by seeding 4x10^6 cells/ml in 75 cm^2 tissue culture flasks and stimulating the cells with the concentrations of mitogens listed above for 6 hrs. Adherent leukocytes were washed three times with HBSS and the cells cultured in serum-free medium for 72 hrs at 20°C. Supernatants were collected, centrifuged (1000 x *g* for 10 min) and filter sterilized. Multiple crude MAF preparations were pooled. MAF raised under these conditions

was used for characterizing the kinetics of nitric oxide production and respiratory burst activity, as well as for purification of the native molecules responsible for MAF activity using FPLC (Chapter VI).

Establishment of macrophage cultures using cell conditioned medium (CCM) containing macrophage growth factor(s) (MGF)

Goldfish macrophage cultures were established by stimulating hematopoeitic-derived kidney leukocytes with CCM containing MGF. Two types of CCM were used as sources of MGF in establishing macrophage cultures: 1) CCM obtained from 7-10 day old GMCL cultures (GMCL-derived CCM); and 2) CCM obtained from 7-10 day old kidney leukocyte cultures (KL-derived CCM).

Bioassay for macrophage growth factor activity in GMCL-derived CCM

Individual wells of a 96 well tissue culture plate were seeded in quadruplicate with 6.25, 12.5, 25, or 50 $x10^3$ kidney leukocytes and induced to proliferate by the addition of varying amounts (v/v) of CCM obtained from 7-to-10 day-old GMCL cultures (0, 6.25, 12.5, or 25% GMCL-derived CCM). Total volume in culture wells was 100 µl. Plates were sealed with Parafilm® and incubated for 8 days at 20°C. Medium was replaced after 4 days of cultivation by gently removing 50% (50 µl) of the medium from each well and replacing it with an equal volume of fresh medium containing the appropriate concentration of GMCL-derived CCM. The medium in the cultures was also changed 12 hr before the assessment of viability using the XTT assay described below.

The kinetics of MGF-induced proliferation of progenitor cells was determined by placing $6.25x10^3$ or $2.5x10^4$ leukocytes into wells of 96 well plates and culturing cells in the absence or presence of GMCL-derived

CCM (final concentration of 25% v/v). The medium in cell cultures was replaced every 4 days (as described above) during the first 8 days of cultivation. Medium was subsequently replaced every 2 days for the duration of the experiment. Cell viability was determined at 0, 4, 8, 12, or 16 days of cultivation using the XTT viability assay.

Bioassay for macrophage growth factor activity in KL-derived CCM

To demonstrate endogenous production of a MGF by kidney leukocytes, leukocytes were seeded in quadruplicate at a density of $1x10^5$ cells/well in 96 well culture plates and the kinetics of growth determined at 0, 2, 4, 8, and 12 days after cultivation using the XTT viability assay (described below). Fresh medium was replaced after 4 days of cultivation and every 2 days thereafter. Supernatants from 0, 2, 4, or 8 day old cultures were removed and tested for their ability to restore the proliferative capability of kidney leukocytes seeded at $1.25x10^4$ kidney leukocytes/well. Thus, $1.25x10^4$ kidney leukocytes were seeded in quadruplicate in wells of a 96 well culture plate in a volume of 75 µl, and induced to proliferate by the addition of 25 µl of conditioned supernatants from the cultures seeded at $1x10^5$ cells/well. Cell number was determined after 12 days of cultivation using XTT viability assay.

XTT Cell Viability Assay

The number of cells in culture wells was indirectly determined using the XTT tetrazolium salt viability assay (Scudiero, et al., 1990; Roehm et al., 1991). Viable goldfish macrophages and leukocytes reduce this compound to a soluble formazan in the presence of an electron coupler (e.g. co-enzyme Q,), with the amount of reduction directly proportional to the number of viable cells in culture (Fig. 2.1). Briefly, 50 µl of medium was removed from individual cultures of 96-well culture plates and replaced with 200 µl of Dulbecco's phosphate buffered saline

containing glucose (DPBS-G). The plates were centrifuged at 100 x *g* for 1 minute, and 200 µl of the supernatant removed. An additional 200 µl of DPBS-G was added to the wells, the plates centrifuged and 200 µl of the supernatant removed from each well. Fifty microlitres of DPBS-G, containing XTT (1 mg/ml) and co-enzyme Q (50 µg/ml), were added to the individual wells and incubated at 20°C for 2 hr. Optical densities were determined at 450 nm using a microplate spectrophotometer (Biotek)

Establishment of *in vitro*-derived kidney macrophage (IVDKM) cultures

IVDKM cultures were established by isolating goldfish kidney leukocytes using 51% continuous Percoll gradient centrifugation, as described above. Leukocytes were cultured in 75 cm^2 tissue culture flasks at concentrations of 5 - 10 x 10^5 cells/mL in 20 ml of medium containing serum, antibiotics, and 25 % filter sterilized GMCL-derived CCM or kidney leukocyte-derived CCM. Leukocytes were incubated for approximately 4-5 days before an additional 5 ml of fresh medium (no CCM) was added to the cultures. Cultures were incubated for an additional 3-6 days before being used in assays. The total duration of cultivation of IVDKM varied from 8-11 days, depending on the growth rates induced by CCM in cultures established from individual fish.

Morphological characterization of goldfish macrophages

Cytochemistry

Cytochemistry was done on cytocentrifuge smears of IVDKM (Cytospin II, Shandon Instruments). Macrophages were stained for non-specific esterase (using α-naphthyl acetate esterase), acid phosphatase, and myeloperoxidase, at 32°C according to the manufacturer's protocols

(Sigma). IVDKM were also stained with a modified Wright's stain (Fisher) for microscopical examination of cell morphology.

Flow cytometric analysis of IVDKM

IVDKM cultures were analyzed by flow cytometry using a FACS Calibur flow cytometer equipped with a cell sorter (Becton Dickinson). Only forward light scatter (size) and side scatter (internal complexity) parameters were considered. All flow cytometric analysis was done in PBS. For experiments involving sorted cell populations, the flow cytometer was first sterilized by passing 10% bleach followed by 70% ethanol through the cytometer. Sorted cells were collected in a 50 ml tube containing 10 ml of medium (with antibiotics and 10% calf serum). Cells were concentrated by centrifuging the suspension (200 x *g* for 10 min) and pooling several tubes of sorted cells. Flow cytometer instrument settings were stored and held constant throughout all experiments. Settings for the flow cytometer were as follows: 1) forward side scatter - photodiode voltage set to E00, AmpGain set to 1.00, and 2) side scatter - photomultiplier voltage set to 480 V, AmpGain set to 1.00.

In some experiments, the number of cells within wells of a 96 well culture plate was determined using flow cytometry. For these experiments, non-adherent cells were removed from wells and the remaining adherent cells trypsinized (0.25% trypsin/ 1mM EDTA) for 3 min. Both adherent and non-adherent cells were placed in polystyrene tubes containing PBS (total volume = 800 µl). Cells were analyzed by flow cytometry at a high flow rate (60 µl/min flow rate) for 3 min. Absolute cell numbers within cultures was determined by taking the number of cells counted by the flow cytometer and multiplying by 4.4 (i.e total tube volume divided by volume analyzed by flow cytometry).

Nitric Oxide Assay

Nitric oxide produced by cytokine and/or LPS-activated macrophages was analyzed by sampling culture supernatants for the presence of nitrite, a stable by-product of nitric oxide, using the Griess reaction (Green et al., 1982). Briefly, 75 µl of macrophage supernatants were removed from individual wells and placed in a separate 96 well microtitre plate. One-hundred microlitres of 1% sulfanilamide in 2.5% phosphoric acid was added to each sample, followed by 100 µl of 0.1% N-naphthyl-ethylenediamine in 2.5% phosphoric acid. Optical density was determined using a spectrophotometer (Biotek) at 540 nm. The molar concentration of nitrite in samples was determined from standard curves generated using known concentrations of sodium nitrite dissolved in medium.

Respiratory Burst Assay

Respiratory burst responses were assayed using the NBT method (Rook et al., 1985). NBT was dissolved in dimethyl sulfoxide (20%w/v) to which pre-warmed (50°C) Dulbecco's PBS (Gibco) was added to completely dissolve NBT (final NBT concentration= 2 mg/ml). This NBT solution was heated for an additional 10 min at 50°C and subsequently filter sterilized. PMA was added to the NBT solution such that the final triggering concentration within macrophage cultures was 50 ng/ml. Fifty µl of the NBT/PMA solution was added to macrophage cultures and incubated for 25 minutes at room temperature. Supernatants were subsequently removed and macrophage layers fixed by adding 200 µl of 70% methanol for 1 minute. Unreduced NBT was removed by washing cells several times with 70% methanol. Reduced NBT was dissolved by

adding 120 µl of 2M KOH to individual wells and pipetting vigorously. One-hundred and forty µl of dimethyl sulfoxide (DMSO) was then added to wells and optical densities read at 630 nm on a microtiterplate spectrophotometer. For half area 96 well culture plates the volumes of methanol, KOH, and DMSO listed were halved.

Pathogens

Mycobacterium chelonei

Mycobacterium chelonei was a kind gift from Dr. James Talbot (Provincial Laboratory of Public Health, University of Alberta). The particular strain was designated 9534 and was originally isolated from liver mesenteric tissues of a fathead minnow. *M. chelonei* causes a disease in teleosts that is histologically similar to tuberculosis in mammals. *M. chelonei* was propagated in Middlebrook 7H9 broth (containing 0.02% Tween 80). Determination of colony forming units (CFUs) was done by plating 1:10 serial dilutions of bacterial suspensions on Middlebrook 7H10 agar plates. The formulation for the various Middlebrook media is listed in Table 2.3.

Aeromonas salmonicida

Aeromonas salmonicida was obtained from Dr. William Paranchych (Department Biological Sciences, University of Alberta). *A. salmonicida* is the causative agent of furunculosis in teleosts. This bacterium was cultured in tryptic soy broth (TSB, Difco) and CFUs determined by plating on TSB agar plates.

Leishmania major

L. major is an obligate intracellular pathogen of mammalian macrophages, and has recently been shown to be capable of infecting goldfish macrophages (Wang et al., 1995). The NIH 173 (WHOM/Ir/-/173) strain of *L. major* was used in the experiments described in this thesis. The promastigote life cycle stage of this parasite was maintained by serial passage in Cunningham's medium (Cunningham, 1977). Viable organisms were determined using a fluorescein diacetate/ethidium bromide viability stain (Belosevic et al., 1988) and enumerated using a haemocytometer. This pathogen was maintained by routine passage through BALB/c mice, as described by Guy (1995).

***Aeromonas salmonicida* phagocytosis assay**

The phagocytic ability of IVDKM was determined using the bacterium *Aeromonas salmonicida*. Bacteria were washed three times in serum free medium by centrifugation (10,000 x *g*, 10 min) prior to addition to macrophages. IVDKM ($5x10^5$ cells) were placed into 6 ml polypropylene tubes (Falcon) in serum free medium and infected with bacteria at a multiplicity of infection of 10:1 (total volume of bacteria and cells was 500 µl in serum free media). Cells were allowed to phagocytose bacteria for 1 hr at 20°C. Non-phagocytosed bacteria were removed by centrifuging the cell suspension three times at 50 x *g* for 10 min. The washed cell pellet was re-suspended in 500µl of serum free medium, smears made by cytocentrifugation (Shandon), and stained using a Modified Wright's stain (Fisher).

Infection of goldfish macrophages with *Leishmania major*

IVDKM or GMCL were seeded into 6 ml polypropylene tubes and infected with *L. major* at various parasite : macrophage ratios (0:1 - 10:1) under serum free conditions. Phagocytosis was allowed to proceed for 3 hrs after which macrophages were centrifuged (200 x *g* for 10 min) and resuspended in medium with serum. In some experiments cytocentrifuge smears were made and the proportion of infected macrophages determined. In other experiments, infected macrophages were cultured for various time periods (0, 24, 48, or 72 hrs) and nitric oxide production determined using the nitric oxide assay described above.

Fast Performance Liquid Chromatography (FPLC)

Preparation of crude MAF samples for FPLC

Batched crude MAF was concentrated by dialysis against PEG flakes (molecular weight = 20 kD). MAF was placed into dialysis bags (molecular weight cutoff = 3 kD, SpectroPor) and placed in a tray of PEG. Concentration was allowed to proceed until approximately one half of the original volume of sample remained in the dialysis bag. Samples were subsequently removed from dialysis bags, pooled together, and re-concentrated. This step-wise concentration of MAF minimized the amount of precipitation of proteins during concentration. MAF samples were concentrated 36 fold using this technique. Concentrated MAF samples were aliquotted into 250 µl samples and stored at -20°C until FPLC analysis was performed.

Gel Permeation FPLC (GP-FPLC)

Concentrated MAF samples were fractionated according to size using a Superose 6 column (Pharmacia). GP-FPLC was carried out at room temperature using an FPLC system from LKB (Pharmacia, Bromma, Sweden). Concentrated MAF samples were either filter sterilized with a 0.22 µM filter (Millipore) or centrifuged at 19,000 x *g* for 10 min before injecting 200 µL fractions onto the column. The running buffer used in GP-FPLC was PBS (pH 7.2). All GP-FPLC purifications were done at flow rates of 0.4 ml/min. Individual fractions were collected at 2.5 min intervals, and upon completion were stored at -20°C until analyzed in MAF activity assays.

Chromatofocusing FPLC (C-FPLC)

Active MAF fractions obtained from GP-FPLC were subsequently fractionated based on their isoelectric points using C-FPLC. Approximately 4 ml of GP-FPLC purified MAF fractions were concentrated using mirocentrifugal concentrators (Filtron). The apparatus consisted of a polystyrene sample chamber separated from a collection chamber by a size exclusion filter (molecular weight cutoff = 3000 D). The polystyrene sample chamber was first blocked for 30 min at room temperature using 10% calf serum to prevent non-specific absorption of MAF activity to the polystyrene sample chamber. Calf serum was diluted in either 0.25 M Tris- pH 7.5, or 0.75M Tris- pH 9.3 depending on the pH gradient used for fractionation (see below). The chamber was washed extensively with Milli-Q-water prior to addition of GP-FPLC purified MAF.

C-FPLC was carried out at room temperature using a Mono-P column (Pharmacia). Proteins were fractionated by chromatofocusing using two different pH gradients: pH 7.5 - 5.0 or pH 9.3 - 6.0. The Mono-P column was pre-equilibrated in 0.025M bis-Tris (balanced to pH 7.5 using 1M HCl) or 0.075M Tris (balanced to pH 9.3 using glacial acetic acid), for

establishment of the upper limits of these gradients, respectively. A linear pH gradient of 7.5 - 5.0 was established by running a 1:10 dilution of Polybuffer 74 (Pharmacia) at a flow rate of 0.75 ml/min through the column. A linear pH gradient of 9.3 - 6.0 was established by running a 1:10 dilution of Polybuffer 96 (Pharmacia) at a flow rate of 0.75 ml/min through the column. MAF samples loaded onto the Mono-P column were allowed to elute through the column for 30 min before initiation of the pH gradient.

C-FPLC fractions were initially collected in tubes containing an equal volume of 10% calf serum (diluted in PBS), in order to stabilize purified MAF activity. MAF activity in these fractionated samples was subsequently determined using the nitric oxide assay described above.

Subsequent purifications using C-FPLC were done in order to characterize fractionated MAF proteins by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). In these experiments, C-FPLC fractions were collected in tubes, and half of the sample removed and added to a tube containing 10% calf serum. These samples were analyzed for MAF activity using the nitric oxide bioassay. Glycerol was added to the remaining sample (50% v/v) and fractions stored at -20°C until analyzed by SDS-PAGE.

SDS-PAGE of GP-FPLC and C-FPLC purified MAF proteins

Proteins purified using FPLC were subsequently analyzed by SDS-PAGE using methods described by Laemmli (1970). Total protein concentrations within fractionated samples was determined using a bicinchoninic protein assay kit (Pierce) according to the manufacturers suggested protocols. FPLC fractionated MAF proteins were dissolved in an equal volume of 2X sample buffer (0.125 M Tris-HCl, pH6.8; 20% glycerol;

4% SDS; 10% 2-β-mercaptoethanol; 0.0025% bromophenol blue), and electrophoresed through 5% stacked, 7.5 - 15% discontinuous polyacrylamide gels. Electrophoresis was carried out at 100 V for 10 min followed by 200 V for approximately 30 min (time required for the bromophenol band to start migrating out of the gel).

Protein bands were visualized by staining the gels with Coomassie Brilliant Blue R-250 (BioRad) dissolved in 40% methanol/10% acetic acid for 1 hr. Gel background was subsequently destained with 40% methanol/10% acetic acid.

Gels stained with Coomassie Brilliant Blue R-250 were also stained using a silver staining kit (BioRad), according to the manufacturers protocols.

Statistics

One and two-way analysis of variance using SuperAnova software (Abacus) for the Power Macintosh was used for determining significance between control and experimental groups. Probability level of $P < 0.05$ was considered significant.

Table 2.1. Composition of medium used to culture goldfish macrophages and leukocytes.[a]

Reagent or Solution	Amount	Special requirements & supplier
Milli-Q®-water	700 ml	endotoxin free, 18MΩ
HEPES	7.00 g	(Sigma)
KH_2PO_4	0.688 g	(BDH)
K_2HPO_4	0.570 g	(BDH)
NaOH	0.75 g	(Fisher)
$NaHCO_3$	0.34 g	(BDH)
Hank's balanced salt solution	80 ml	10X solution, no Mg^{2+} or Ca^{2+}
MEM amino acid solution	25 ml	50X (Gibco)
MEM non-essential amino acids	25 ml	100X (Gibco)
sodium pyruvate	25 ml	100X (Gibco)
MEM vitamin solution	20 ml	100X (Gibco)
nucleic acid solution	20 ml	solution containing 2.5 mM each of adenosine, cytidine, hypoxanthine, thymidine, and uridine (Sigma)
L-glutamine	0.584 g	(Sigma)
2-β-mercaptoethanol	0.007 ml	(Sigma)
insulin	0.010 g	(Sigma)
GFL-15 medium	1000 ml	Base medium comprised of equal volumes of DMEM (high glucose, no pyruvate, no sodium bicarbonate, Gibco) and Leibovitz's medium (Gibco).

[a]Reagents were added in the order listed. Medium was balanced to pH 7.4 using 5M NaOH, and the total volume brought up to 2 litres.

Table 2.2. Reagent composition of Middlebrook media used to cultivate *M. chelonei* (9534).[a]

	Middlebrook 7H9 Broth	Middlebrook 7H10 agar
ammonium sulfate	0.05 g	0.05 g
monopotassium phosphate	1.0 g	1.0 g
disodium phosphate	2.5 g	2.5 g
sodium citrate	0.1 g	0.4 g
magnesium sulfate	0.05 g	0.025 g
calcium chloride	0.0005 g	0.0005 g
zinc sulfate	0.001 g	0.001 g
copper sulfate	0.001 g	0.001 g
L-glutamic acid	0.5 g	0.5 g
sodium chloride	-	0.85 g
ferric ammonium citrate	0.04 g	0.04 g
pyridoxine	0.001 g	0.001 g
biotin	0.0005 g	0.0005 g
malachite green	-	0.00025 g
glycerol	2 ml	5 ml
bacto agar	-	15 g
oleic acid	-	0.05 g
bovine albumin (fraction V)	5 g	5 g
dextrose	2 g	2 g
catalase	0.003 g	0.004 g

[a]Medium was prepared by adding all of the reagents, except the last four, to 900 ml of distilled water. This solution was balanced to pH 6.6, and sterilized by autoclaving. The last four reagents were added to 100 ml of distilled water, 0.22 µM filter sterilized and added to the other reagents.

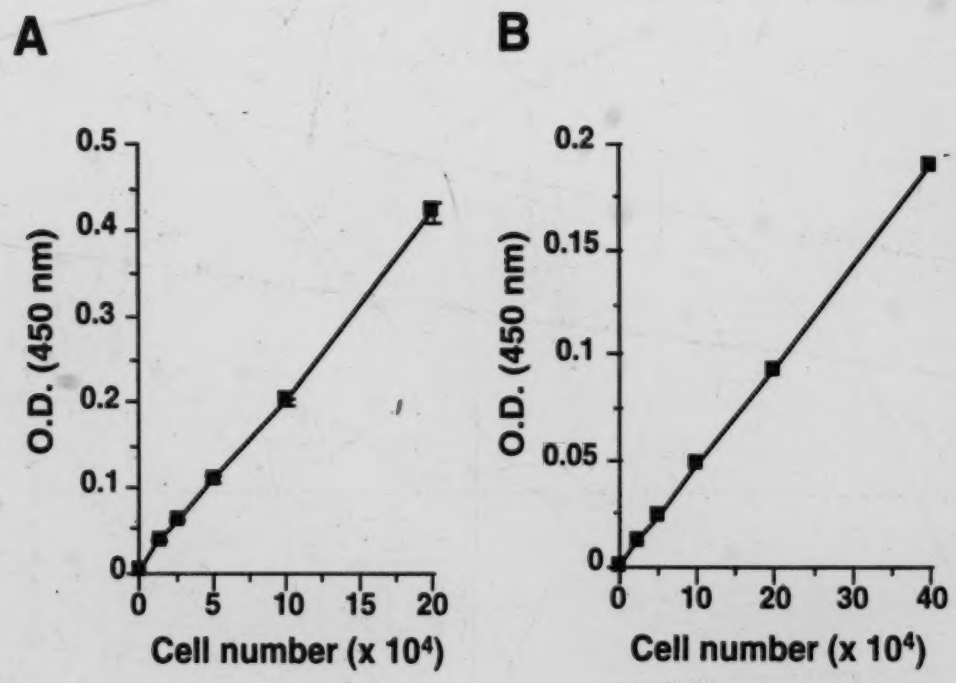

Figure 2.1. The reduction of the tetrazolium salt XTT to a soluble formazan in the presence of co-enzyme-Q by GMCL (A) and kidney leukocytes (B). The colorimetric reaction (O.D. 450 nm) is directly proportional to the number of viable cells within culture. Each point represents the mean ± SEM of quadruplicate cultures for GMCL and mean ± SEM of quadruplicate cultures obtained from kidney leukocytes of 4 fish.

CHAPTER III

ESTABLISHMENT AND CHARACTERIZATION OF GOLDFISH MACROPHAGE CULTURES [1]

Introduction

Macrophages are pivotal cells that mediate facets of cellular and humoral immunity in vertebrates, and their continuous renewal and differentiation into functionally mature effector cells is of vital importance in host defense. The mononuclear phagocyte system theory, proposed by van Furth and colleagues (1972), has been widely accepted as an explanation for macrophage hematopoeisis in higher vertebrates. This theory describes tissue macrophages as ubiquitously distributed, terminally differentiated cell populations having limited proliferative capability. Expired populations of tissue macrophages are restored by monocytes that migrate into tissues from the circulation, and which subsequently undergo site-specific maturation. Monocytes, in turn, originate from progenitor cells in the bone marrow, known as promonocytes, the latter of which presumably develop from monoblasts. Monoblasts are the primordial committed cell in the macrophage lineage, differentiating from granulocyte-macrophage colony forming cells. These bi-potential progenitors are also capable of differentiating towards the neutrophil lineage, and thus represent an important regulatory point in the development of both neutrophils and macrophages in higher vertebrates (van Furth 1981; Cannistra and Griffin, 1988; Stewart et al., 1994; Metcalf and Nicola, 1995) .

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[1] A version of this chapter has been published: Neumann, et al., 1998. *Dev. Comp. Immunol.*, 22: 417-432.

There is evidence, however, that challenges this concept of macrophage renewal. Tissue macrophages may represent a unique population of cells, whose numbers are maintained within tissues through the process of self-renewal. For example, the elimination of circulating monocytes, by strontium treatment, does not affect the total numbers or proliferative potential of resident peritoneal macrophages, alveolar macrophages, or Kupffer cells (Sawyer et al., 1982; Shibata et al., 1985; Yamamda et al., 1990). Moreover, fetal macrophages that eventually colonize the fetal liver appear early in embryonic development, prior to the formation of bone marrow (Naito et al., 1996; Yamashita et al., 1996). The most important evidence supporting the self-renewal of tissue macrophages comes from studies addressing renewal of microglia in the central nervous system. Microglia-specific progenitor cells have been identified within the brain, a site not readily available for re-population via influx of monocytes (Alliot et al., 1991). These data suggest that resident macrophages in certain tissues may be unique populations of self replicating cells, and not derived through normal hematopoietic pathways.

Hematopoiesis is a highly regulated event, maintaining steady state levels of blood cells during homeostasis, and upregulating specific cell populations when homeostasis is perturbed (e.g. infection or anemia). Macrophage hematopoiesis is upregulated in response to immune challenge (Ralph, 1989). By far the most important cytokine involved in the proliferation of macrophages in mammals is macrophage colony stimulating factor (M-CSF or CSF-1). M-CSF acts on all progenitor cells of the macrophage lineage, directing cell differentiation towards monocyte development. In fact, macrophage progenitor cells (monoblasts and promonocytes), are dependent on the presence of this cytokine for growth and viability (Rutherford et al., 1993). *In vitro* cultivation of murine bone marrow cells in the presence of macrophage growth factors, such as M-CSF

(derived from fibroblasts), results in the selective proliferation and differentiation of macrophages (Neumann and Sorg, 1980; Falk et al., 1988; Belosevic et al., 1988; Metcalf and Nicola, 1995). Other growth factors such as IL-3, IL-6, and GM-CSF, also play important accessory roles in channeling hematopoiesis towards macrophage development (Donahue et al., 1988; Jansen et al., 1992; Falk and Vogel, 1988; Ikebuchi et al., 1987; Caracciolo et al., 1987; Falk et al., 1988; Metcalf, 1980; Metcalf and Burgess, 1982).

Although macrophage specific hematopoiesis has been extensively characterized in mammals, no information of this phenomenon is available for fish. We have recently established a cell line from goldfish kidney hematopoeitic tissues which shows morphological, cytochemical, and functional similarities to macrophages (Wang et al., 1995). These cells are: 1) similar to goldfish kidney macrophages as determined by light and transmission electron microscopy; 2) adherent to plastic and glass; 3) able to form multinucleated giant cells in culture; 4) non-specific esterase positive; 5) chemotactic; 6) phagocytic against sheep red blood cells and *Leishmania major*, an obligate intracellular pathogen of mammalian macrophages; and 7) potent producers of reactive oxygen and nitrogen intermediates in response to stimulation with cytokines and/or bacterial LPS.

This spontaneously arising goldfish macrophage cell line has been propagated in our laboratory for the past 6 years. The propagation of this cell line in culture appears to be dependent on autocrine production of MGF, since GMCL require the presence of supernatants from actively growing cultures in order to proliferate *in vitro* (Wang et al., 1995).

This chapter describes the use of GMCL and kidney leukocyte-derived MGF for establishment of primary macrophage cultures from goldfish kidney hematopoeitic tissues. The characterization and establishment of goldfish macrophage cultures was an important step in

my attempts to define the regulatory mechanisms governing macrophage activation in teleosts.

Results

Production of MGF by GMCL

Primary goldfish macrophage cultures were established *in vitro* using leukocytes isolated from kidney hematopoeitic tissues of the goldfish, as described in Chapter II. Kidney leukocytes were stimulated to proliferate and differentiate in culture by adding CCM obtained from 7-10 day old GMCL cultures.

Goldfish kidney leukocytes cultured in the presence of GMCL-derived CCM proliferated significantly than leukocytes cultured in the absence of supernatants, provided that relatively few kidney leukocytes were used for the initial seeding inoculum in the cultures (Fig. 3.1). Thus, wells seeded with $6.25x10^3$ or $1.25x10^4$ cells and stimulated with 25% (v/v) GMCL-derived CCM, had significantly more cells after 8 days of cultivation than did control leukocyte cultures not supplemented with these supernatants ($P < 0.05$). The presence of GMCL-derived CCM did not significantly alter cell number after 8 days of cultivation if higher densities of kidney leukocytes ($5x10^4$ cells) were initially seeded into the wells ($P > 0.05$), indicating that goldfish kidney leukocytes, seeded above a threshold density, may produce sufficient endogenous MGF for induction of cell proliferation and differentiation *in vitro*. The proliferation-inducing activity of GMCL-derived CCM on kidney leukocyte cultures was also dose dependent (Fig. 3.2).

Production of endogenous MGF by goldfish kidney leukocytes

Corroborating evidence for endogenous production of a kidney leukocyte-derived MGF was obtained by examining the kinetics of proliferation of these cells in the presence or absence of GMCL-derived CCM. Kidney leukocytes seeded at densities of $2.5x10^4$ cells/well proliferated over 16 days of culture in the presence or absence of GMCL-derived CCM (Fig. 3.3). However, significantly greater numbers of cells were present in cultures treated with GMCL-derived CCM after 12 and 16 days of cultivation compared to controls ($P < 0.05$ for both days, Fig 3.3). Leukocytes cultured at densities of $6.25x10^3$ cells/well, failed to proliferate without the addition of exogenous GMCL-derived CCM over this same time period (Fig. 3.3).

When $1x10^5$ kidney leukocytes were seeded into wells of a 96 well tissue culture plate, cells proliferated in the absence of exogenous growth factor (Fig. 3.4). Moreover, the supernatants obtained from these cultures significantly enhanced the proliferation of kidney leukocytes seeded at $1.25x10^4$ cells/well, providing direct evidence for endogenous production of MGF by kidney leukocytes (Fig. 3.4). Growth-enhancing supernatants obtained from kidney leukocytes are termed kidney leukocyte-derived cell conditioned medium (KL-derived CCM).

Characterization of primary macrophage cultures from goldfish

The predominant cell type in kidney leukocyte cultures grown in the presence of GMCL-derived CCM or KL-derived CCM were characterized as macrophages, based on the following criteria: 1) flow cytometric profile, 2) morphology and cytochemistry, 3) phagocytic ability, and 4) production of reactive oxygen and nitrogen intermediates.

Macrophage cultures established using these growth-enhancing supernatants are termed *in vitro*-derived kidney macrophages (IVDKM).

Flow cytometric profile of IVDKM

Analysis of IVDKM by flow cytometry (using size and internal complexity as discriminating parameters), demonstrated that three distinct sub-populations of cells were present in IVDKM cultures after 8 days of cultivation (Fig. 3.5). Interestingly, GMCL cultures also had a similar cytometric profile (Fig. 3.6), suggesting that the sub-populations of cells observed in IVDKM and GMCL cultures may represent macrophages developmentally arrested at distinct differentiation junctures in the teleost macrophage lineage. The three sub-populations of macrophages in IVDKM and GMCL cultures were designated R1, R2, and R3-type macrophages. The relative proportions of macrophages comprising these sub-populations in IVDKM cultures is given in Table 3.1.

Flow cytometric analysis was also done on kidney leukocyte cultures after 1, 2, 4, or 8 days of cultivation, to determine a chronologic sequence of cell development in IVDKM cultures. After 1 day in culture, the majority of cells were found in region 1, with few cells scattered in regions 2 and 3 (Fig. 3.7). During the next 4 days of cultivation, increasing numbers of cells began to appear in region 2, and by day 4 a distinct focus of cells was observed in region 2 (Fig. 3.7). With respect to R3-type macrophage development, a focus of cells within region 3 was observed during the first two days of cultivation; however this focus of cells appeared to dissipate by 4 days of cultivation. A distinct focus of cells within region 3 then re-appeared after 8 days of cultivation (Fig. 3.7).

These three macrophage sub-populations were subsequently sorted using the flow cytometer, and their proliferative potential and differentiation patterns examined further. R1-type macrophages proliferated in response to KL-derived CCM, and appeared to differentiate

into R2-type macrophages over 4-8 days of cultivation (Fig. 3.8 and Table 3.2). Their proliferation and differentiation into R2-type macrophages was not dependent on the addition of exogenous growth factor, in that R1-type macrophages cultured in medium only also proliferated and differentiated into R2-type cells (Fig. 3.8). However, proliferation of unstimulated R1-type macrophages was slower compared to those stimulated with KL-derived CCM (Table 3.2).

R2-type macrophages appeared to be a more mature sub-population of macrophage, in that sorted R2 cells remained in their sort region after 4 days of cultivation in the presence or absence of exogenously added KL-derived CCM (Fig. 3.9). Interestingly, this sub-population of macrophage proliferated in culture in the presence of growth enhancing supernatants (Table 3.3). Moreover, in one of the trials, R2-type macrophages also proliferated in the absence of CCM (Table 3.3); however, proliferation occurred sooner and was greater in R2-type cell cultures stimulated with KL-derived CCM when compared to unstimulated cultures (Table 3.3).

R3-type macrophages appeared to be temporally arrested in macrophage development in IVDKM cultures. Sorted R3-type macrophages appeared to differentiate into R2-type macrophages, provided that they were cultured in the presence of KL-derived CCM (Fig. 3.10). R3-type macrophages cultured in the absence of KL-derived CCM died (Fig. 3.10 and Table 3.4), indicating that the MGF present in KL-derived CCM sustains the viability of this macrophage sub-population *in vitro*. Interestingly, R3-type macrophages appeared to differentiate into R2-type macrophages when stimulated with MAF or LPS (Fig. 3.11). The ability of these cells to differentiate into R2-type macrophages in response to stimulation with LPS alone, suggests that the molecule(s) responsible for their differentiation may be of autocrine origin.

Morphological, cytochemical, and functional characteristics of IVDKM

Morphologic and cytochemical characteristics

IVDKM cultures consisted of both non-adherent and adherent cell populations (Fig. 3.12), with non-adherent cells comprising the vast majority of cells within IVDKM cultures ($5x10^5$ to $1x10^6$ cells/ml). The adherent cell population displayed typical macrophage morphology; spreading extensively on tissue culture flasks ('fried-egg' appearance), and in many cases forming multinucleated giant cells (Fig 3.12). Interestingly, the flow cytometric profile of adherent and non-adherent cells was similar (Fig. 3.13).

The three sub-populations of macrophages seen in IVDKM cultures were sorted and their morphological and cytochemical characteristics examined. R1-type macrophages were small cells (6-10 µm in diameter) with a high nucleus to cytoplasm ratio (Fig. 3.14). These cells stained positively for acid phosphatase, but did not stain for myeloperoxidase or non-specific esterase (Figs. 3.15 - 3.17).

R2-type macrophages were large irregularly shaped cells (~12-20 µm in diameter), with a low nucleus to cytoplasm ratio, and often had extensive vacuolization and membrane ruffling (Fig. 3.14). R2-type cells stained positively for acid phosphatase, and had localized staining for myeloperoxidase (Figs. 3.15 and 3.16). These cells showed variability in staining for non-specific esterase (Fig. 3.17).

R3-type macrophages were large round cells (~12-15 µm in diameter) with a low nucleus to cytoplasm ratio, and often had eccentrically placed "kidney" shaped nuclei (Figs. 3.14 and 3.16). The morphologic appearance of these cells was similar to that of monocytes. Interestingly, a human monocyte-like cell line, U937, segregated to the same region as R3-type macrophages using the identical flow cytometer settings for analyzing IVDKM (Fig. 3.18). R3-type macrophages were acid

phosphatase positive, myeloperoxidase positive, and showed variability in staining for non specific esterase (Figs 3.15 - 3.17).

Functional characteristics

IVDKM were extremely phagocytic, capable of ingesting large numbers of bacteria (Fig. 3.19). Moreover, both R2 and R3-type macrophages actively phagocytosed *Leishmania major*, with 17% of R3-type macrophages and 14% of R2-type macrophages becoming infected after a 3 hr period (10:1 parasite to macrophage ratio). R1-type macrophages were not phagocytic (<0.5% infected), although parasites could often be seen adhering to the cell surface of R1-type macrophages

IVDKM produced both reactive oxygen and nitrogen intermediates in response to stimulation with cytokines and/or pathogen-derived molecules. The production of these reactive intermediates by IVDKM is characterized extensively in Chapters IV, V, and VII of this thesis.

Discussion

Renewal of effete macrophage populations, and increased production of macrophages during infection is essential for sustaining immune surveillance and competence. Proliferation and differentiation of macrophages is a highly regulated event, dependent upon the concerted actions of several growth factors. Although much is known about the mechanisms of macrophage development in mammals, little is known about these events in lower vertebrates, including fish.

My results demonstrate that goldfish macrophages and kidney leukocytes secrete MGF that induce proliferation and differentiation of macrophages from hematopoietic tissues of the goldfish. These findings are similar to those observed for mammalian macrophages, where the

conditioned supernatants of cell cultures obtained from different organs have been shown to induce macrophage colony formation of mammalian bone marrow cultures (Bradley and Metcalf, 1966; Metcalf et al., 1967; Metcalf, 1968; Metcalf, 1970; Burgess et al., 1977; Cutler et al., 1985). Spontaneous macrophage proliferation occurs in mammalian bone marrow cultures seeded with high numbers of cells, a result of localized production of macrophage growth factors by stromal cells close to responsive progenitor cells (Bradley and Metcalf, 1966; Metcalf et al., 1967; Chan and Metcalf, 1972).

My data suggest that goldfish macrophages may act as a cellular source of MGF, and that the hematopoietic pathways controlling the development of macrophages in teleosts may be different from those of mammalian macrophages. It is well established that mammalian macrophages do not secrete appreciable levels of autocrine growth factors to support their own growth in tissue cultures prepared from hematopoietic organs, and are dependent upon production of these mediators by other cells such as fibroblasts, T-cells, and endothelial cells (Bagby et al., 1983 a,b; Broudy et al., 1986 a,b; Munker et al., 1986; Zucali et al., 1986). At present, my experiments cannot conclusively rule out the possibility that goldfish macrophages secrete soluble factors that upregulate production of MGF by other cell types in our leukocyte cultures, as is the case for mammalian macrophages (Bagby et al., 1983 a,b; Broudy et al., 1986 a,b; Munker et al., 1986; Zucali et al., 1986). However, propagation of our goldfish macrophage cell line *in vitro* requires a certain amount of conditioned medium (i.e. supernatants from previous cultures) to be added to new cultures to maintain *in vitro* growth of this line (Wang et al., 1995; my personal observations). Complete removal of conditioned medium and replacement with fresh medium, or sub-cultivation of macrophages at low densities, invariably results in the failure of cells to proliferate and differentiate *in vitro* (Wang et al., 1995;

Neumann & Belosevic, unpublished observations). This phenomenon has also been observed by others. Gannassin and Bohls (1998) have demonstrated that a macrophage-like cell line, established from rainbow trout, requires supernatants from previous cultures for continuous passage. Moreover, they have demonstrated that this cell line actively proliferates in response to its own CCM. Taken together, these data suggest that teleost macrophages may directly mediate self-propagation and renewal in fish. At present, it is not known which macrophage sub-population (i.e. R1, R2, or R3-type macrophages) mediates cellular proliferation in IVDKM cultures.

This hypothesis is further supported by the uncommon frequency at which spontaneously arising macrophage cell lines, or long term primary macrophage cultures, can be established *in vitro* from fish (Vallejo et al., 1992; Miller et al., 1994; Wang et al., 1995; Gannassin and Bohls, 1996; Dannevig et al., 1997; Weyts et al., 1997; Gannassin and Bohls, 1998). Interestingly, long term macrophage cultures can be readily established from the blood of channel catfish (*Ictalurus punctatus*), suggesting that blood monocytes in fish may have high proliferative potential (Vallejo et al., 1992). Although the macrophage-growth enhancing effects of these cell lines or cultures have not been examined, proliferation *in vitro* may be dependent on autocrine mediators released into the medium of these cells, an effect similar to that seen for GMCL.

At present, it is difficult to conclusively determine whether fish macrophages mediated their own propagation and renewal, since none of the spontaneously arising cell lines, mentioned above, are truly clonal in nature. As such, it is plausible that small numbers of contaminating cells contribute to the macrophage growth-enhancing effects observed in the supernatants of these cell lines. Analysis of the roles that fish macrophage growth factors play in modulating hematopoiesis in fish must await the availability of purified or recombinant growth factors.

The kinetics of IVDKM proliferation in culture are very similar to those reported for mammalian macrophages established from bone marrow-derived cell cultures in the presence of fibroblast-derived conditioned medium (Neumann and Sorg, 1980). IVDKM cultures undergo a lag phase of growth during the first 4-5 days of cultivation, where cell proliferation is minimal and where non-proliferating or short-lived cells die, as indicated by the extensive cell debris present in these cultures. However, debris is rapidly cleared by the remaining cells, and over the next 4 days of cultivation, a significant increase in the number of cells occurs. Both adherent and non-adherent cells are present in these cultures: adherent cells spread extensively on tissue culture flasks and often form multinucleated giant cells. The non-adherent cells grow in cell clusters and attain significant cell densities in the culture ($> 5 \times 10^5$ to 2×10^6 cells/mL). After 8 days of cultivation, the predominant cell type in the culture is the macrophage, based on the following criteria: (1) flow cytometric profile (i.e. similar flow cytometric profile as GMCL); 2) morphological and cytochemical characteristics; (3) phagocytic ability; and (4) production of reactive oxygen and nitrogen intermediates.

IVDKM represent a heterogeneous population of cells, as determined by flow cytometry, morphology, cytochemistry, and physiological function. Three distinct macrophage sub-populations were observed in both IVDKM and GMCL cultures using flow cytometry, suggesting that these sub-populations of cells may represent macrophages arrested at distinct differentiation junctures in development. Evidence for this hypothesis was obtained by examining the proliferative and differentiation potentials of these purified cell populations using flow cytometry.

R1-type macrophages were small cells and appeared to be progenitors of the more mature R2-type macrophage. Their size and cytochemical profile suggest that these cells may be similar to the

mammalian monoblast (Beelen et al., 1989; Stachura, 1989). R1-type macrophages contained acid phosphatase, but lacked myeloperoxidase and non-specific esterase activity. R1-type macrophages proliferated, and eventually differentiated into R2-type macrophages, the rates of which were enhanced in the presence of KL-derived CCM. The ability of R1-type macrophages to proliferate and differentiate in the absence of exogenous growth factors suggests that these cells may be autocrine producers of MGF in teleosts.

R2-type macrophages were morphologically similar to mammalian tissue macrophages, having an abundant cytoplasm, irregular shape, and extensive vacuolization. These cells contained acid phosphatase, but showed variable non-specific esterase activity. They also possessed myeloperoxidase, the presence of which was restricted to specific foci within the cell. Thus, as R1-type macrophages differentiate into R2-macrophages they appear to acquire both myeloperoxidase and non-specific esterase activity. The acquisition of myeloperoxidase activity in the more mature R2-type macrophage is unusual, in that the expression of this enzyme in mammalian macrophages is different. As mammalian macrophage progenitors mature, they lose myeloperoxidase activity (Beelin et al., 1989; Stachura, 1989). The acquisition of non-specific esterase activity in R2-type macrophages also appears to be unique in fish, since this enzyme is often used as a cytochemical marker of mammalian macrophages and their progenitors (Stachura, 1989). It is important to note that non-specific esterase activity in IVDKM can be extremely variable. In some cases, IVDKM cultures that displayed weak non-specific esterase activity often had extremely potent nitric oxide responses (Neumann et al., 1998). Moreover, macrophages found in cultures that stained weakly for non-specific esterase were morphologically indistinguishable from macrophages obtained from cultures displaying strong non-specific esterase activity (Neumann et al., 1998). It has been

demonstrated in mammals that non-specific esterase can be an acquired characteristic in the development of murine bone marrow-derived macrophages (Neumann and Sorg, 1980; Stachura, 1989). Moreover, there is often a heterogeneous expression of this enzyme in different mature mammalian macrophage sub-populations (Becker et al., 1983; Stachura, 1989), suggesting that it may not be a definitive cytochemical marker of macrophages. Functionally, R2-type macrophages are highly phagocytic, and potent producers of reactive oxygen and nitrogen intermediates when stimulated with cytokines and/or LPS (Chapters IV and V). Interestingly, this sub-population of macrophage appears to be capable of self replication. Although these cells self replicate, their absolute proliferative potential (i.e. number of sequential cell divisions) is not known. Mature tissue macrophage populations in mammals can be induced to proliferate, although their proliferation potential is less than that of their precursors (Stewart et al., 1994; Metcalf and Nicola, 1995). What is particularly interesting with respect to the development of R2-type goldfish macrophages, is that they can arise without first developing into R3-type macrophages (i.e. putative monocyte). These data suggest that certain sub-populations of mature teleost macrophages may not be renewed via hematopoeisis, but rather, may depend on self renewal or differentiation of a tissue specific progenitor cells (i.e. R1-type macrophage). There is evidence in the literature to suggest that both self renewal and differentiation of tissue specific progenitor cells may account for the renewal of some mature macrophage populations in mammals (Sawyer et al., 1982; Shibata et al., 1985; Alliot et al., 1991; Stewart et al., 1994). It is therefore plausible that multiple pathways of macrophage development occur within IVDKM cultures: 1) through normal hematopoeitic pathways, 2) through immediate progenitor development, and 3) by self-replication.

R3-type macrophages appeared to be temporally arrested in macrophage development in IVDKM cultures. These cells were acid phosphatase and myeloperoxidase positive, but showed variability in non-specific esterase activity. Myeloperoxidase activity in this sup-population of macrophage was different than that observed in R2-type macrophages. Myeloperoxidase was evenly distributed throughout the cytoplasm of R3-type macrophages, as opposed to a more localized distribution in R2-type macrophages. R3-type macrophages were round cells with eccentrically placed kidney shaped nuclei, were phagocytic, and produced reactive oxygen intermediates when stimulated with cytokines and/or LPS (Chapter V). Their morphological, cytochemical, and functional characteristics resemble that of mammalian monocytes. Interestingly, a human monocyte-like cell line (U937) segregated to the same region as R3-type macrophages when analyzed by flow cytometry, providing further evidence that these cells may be monocytic in nature. R3-type macrophages were dependent upon the presence of KL-derived CCM for viability, but were incapable of proliferating. However, this sub-population of macrophage appeared to differentiate into R2-type macrophages when cultured in the presence of KL-derived CCM, MAF, or LPS. Since diffferentiation of R3->R2-type macrophages was induced by stimulation with LPS alone, it would appear that R3-type macrophages may be autocrine producers of their own differentiation factor(s).

It is not known where or how R3-type macrophages originate in IVDKM cultures. Although R1-type macrophages appear to be progenitors of R2-type macrophages, it is plausible that this precursor population may be induced to differentiate into R3-type macrophages. Their differentiation into R3-type macrophages may be dependent upon membrane bound growth factors. Thus, cell-to-cell contact between R1 and R3-type macrophages may be a pre-requisite for driving differentiation toward R3-type macrophage development. This hypothesis would explain

why sorted R1-type macrophages were only capable of differentiation into R2-type macrophages. There is evidence to suggest that membrane bound growth factors play important roles in macrophage development in mammals (Metcalf and Nicola, 1995).

Alternatively, secretory products produced by R2-type macrophages may be required for the development of R3-type macrophages. This hypothesis would account for the observation that a well defined R2-type macrophage sub-population appears in IVDKM cultures before the appearance of a R3-type sub-population. Figure 3.19 summarizes my observations of macrophage development in IVDKM cultures.

Based on the experiments outlined in this chapter, it is difficult to conclusively define the sequence of macrophage development in IVDKM cultures. It is possible that progenitor cells, having high proliferative potentials, that inadvertently segregate to the various sort regions during flow cytometry, contribute to the patterns of macrophage development observed. Thus, the progression of cell development observed in a specific sub-population of macrophage (i.e. R1 -> R2-type macrophages) may not necessarily represent differentiation of that sub-population, but may be explained by the rapid proliferation of a progenitor cell found within the sort region. To conclusively demonstrate the patterns of macrophage development, future experiments will require that the proliferative potentials and proliferative rates (i.e. generation times) of individual macrophages within these sub-populations be determined. This can be done by conducting limiting dilution assays, in which a single macrophage from a sorted sub-population can be cultured over time, and its proliferative potential and proliferative rates determined. Moreover, future experiments should also focus on the production of monoclonal antibodies directed against potential stage-specific antigens found on the surface of the different macrophage sub-populations. It remains to be

determined whether different macrophage sub-populations in IVDKM cultures express unique marker molecules on their surface.

Table 3.1. Proportion of R1, R2, and R3-type macrophages in IVDKM cultures.[a]

Macrophage sub-population	Percent of IVDKM culture (±SEM)	Range (%)
R1	13.1 ± 0.9	8.0 - 17.0
R2	27.6 ± 2.6	14.7 - 44.7
R3	42.2 ± 2.5	32.0 - 58.0

[a] Results obtained from ten individual IVDKM cultures analyzed by flow cytometry.

Table 3.2. Proliferative potential of R1-type macrophages[a].

Culture Conditions	Days in Culture	#macrophages/well ± SEM ($x10^4$)
KL-derived CCM present	0	1.24 ± 0.101
	2	0.94 ± 0.001
	4	1.21 ± 0.060^{c}
	6	$2.38 \pm 0.195^{b,c}$
	8	$4.42 \pm 0.237^{b,c}$
KL-derived CCM absent	0	1.22 ± 0.068
	2	0.88 ± 0.039
	4	0.85 ± 0.060^{b}
	6	0.90 ± 0.251^{b}
	8	3.14 ± 0.046^{b}

[a] R1 type macrophages were sorted using flow cytometry and seeded into wells of half area 96 well culture plates (approximately $1.25x10^4$ cells). The cells were cultured in the presence or absence of KL-derived CCM. The number of macrophages within duplicate wells was determined on the specified days using flow cytometry.

[b] The number of macrophages within culture is significantly different than that observed on day 0 of the same treatment group ($P < 0.05$).

[c] The number of macrophages within the KL-derived CCM treated group is significantly different than the number of macrophages within control cultures at that same time point ($P < 0.05$).

Table 3.3. Proliferative potential of R2 type macrophages.[a]

Trial #	Culture Conditions	Days in Culture	#macrophages/well ± SEM ($x10^4$)
1	KL-derived CCM present	0	2.50[b]
		2	3.56 + 0.138[c,d]
		4	3.93 + 0.251[c,d]
	KL-derived CCM absent	0	2.50[b]
		2	2.25 + 0.082
		4	3.12 + 0.007[c]
2	KL-derived CCM present	0	2.56 + 0.093
		2	2.58 + 0.170
		4	3.75 + 0.040[c,d]
	KL-derived CCM absent	0	2.56 + 0.093
		2	2.53 + 0.133
		4	2.73 + 0.060

[a]R2 type macrophages were sorted using flow cytometry and seeded into wells of half area 96 well culture plates (approximately $2.5x10^4$ cells). The cells were cultured in the presence or absence of KL-derived CCM. The number of macrophages within duplicate wells was determined on the specified days using flow cytometry.

[b]Initial seeding densities determined using a hemocytometer.

[c] The number of macrophages within culture is significantly different than that observed on day 0 of the same treatment group ($P < 0.05$).

[d] The number of macrophages within the KL-derived CCM treated group is significantly different than the number of macrophages within control cultures at that same time point ($P < 0.05$).

Table 3.4. Proliferative potential of R3 type macrophages.[a]

Culture Conditions	Days in Culture	#macrophages/well ± SEM ($x10^4$)
KL-derived CCM present	0	2.50[b]
	2	2.52 + 0.056[d]
	4	2.34 + 0.133[d]
KL-derived CCM absent	0	2.50[b]
	2	2.23 + 0.043
	4	1.83 + 0.034[c]

[a]R3 type macrophages were sorted using flow cytometry and seeded into wells of half area 96 well culture plates (approximately $2.5x10^4$ cells). The cells were cultured in the presence or absence of KL-derived CCM. The number of macrophages within duplicate wells was determined on the specified days using flow cytometry.

[b]Initial seeding densities determined using a hemocytometer.

[c] The number of macrophages within culture is significantly different than that observed on day 0 of the same treatment group ($P < 0.05$).

[d] The number of macrophages within the KL-derived CCM treated group is significantly different than the number of macrophages within control cultures at that same time point ($P < 0.05$).

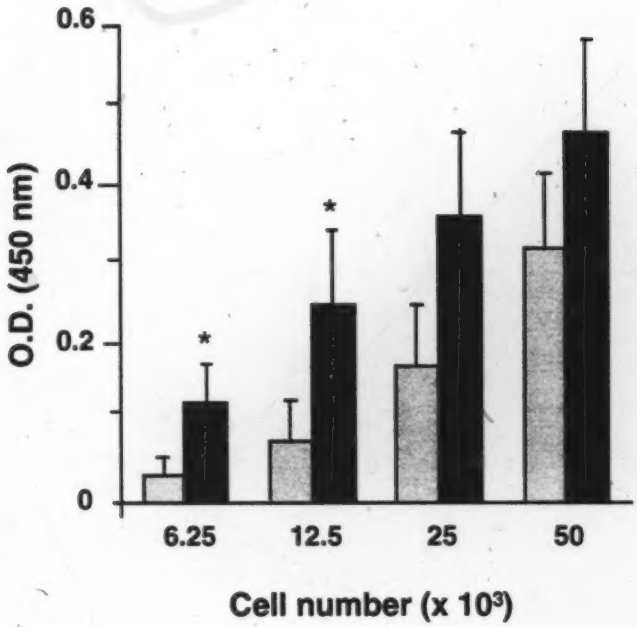

Figure 3.1. Induction of proliferation of goldfish kidney leukocytes by GMCL-derived CCM. Varying numbers of kidney leukocytes were seeded in wells of a 96 well culture plate and cultured in the presence (black) or absence (gray) of 25% (v/v) GMCL-derived CCM. Cell viability was determined after 8 days of cultivation using the XTT viability assay. Each bar represents the mean ± SEM of quadruplicate cultures established from 8 fish. (*)$P < 0.05$, comparing presence and absence of GMCL-derived CCM in that same treatment group.

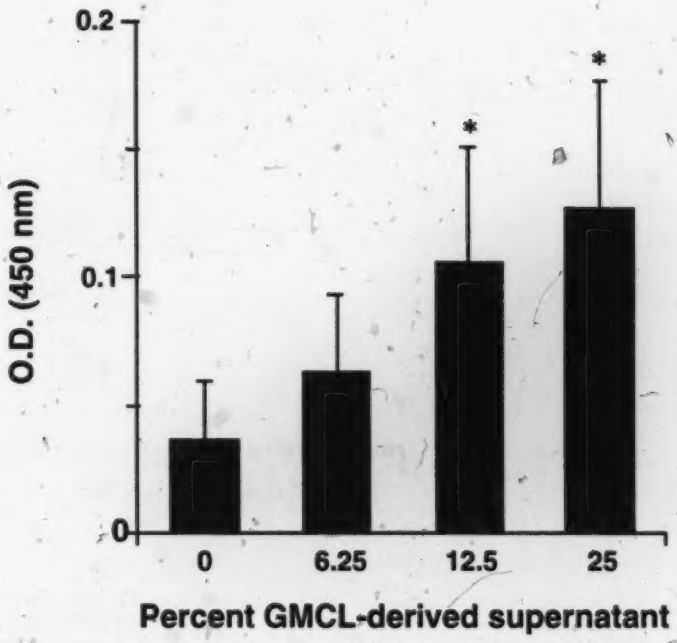

Figure 3.2. Dose dependency of kidney leukocyte proliferation on GMCL-derived CCM. Kidney leukocytes ($6.25x10^3$ cells) were seeded into 96 well culture plates and stimulated to proliferate using different concentrations of GMCL-derived CCM (%v/v). Cell viability was determined after 8 days of cultivation using the XTT viability assay. Each bar in the above graph represents the mean ± SEM of quadruplicate cultures established from 8 fish. (*)$P < 0.05$, when comparing control to GMCL-derived CCM treated groups.

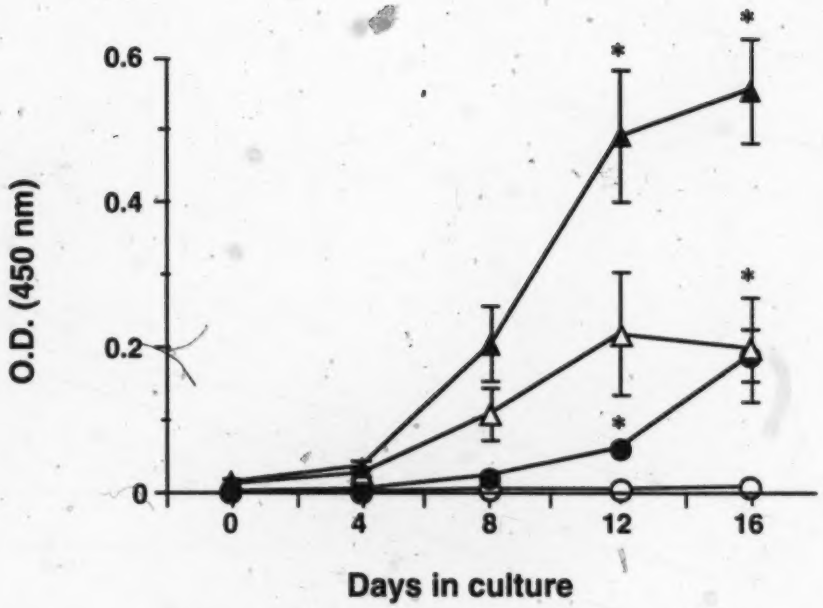

Figure 3.3. Proliferation of kidney leukocytes cultured in the presence or absence of GMCL-derived CCM. Wells of a 96-well culture plate were seeded with 6.25 x10^3 kidney leukocytes (circles) or 2.5x10^4 kidney leukocytes (triangles), and cultured in the absence (open symbols) or presence of 25% (v/v) GMCL-derived CCM (filled symbols). Cell viability was determined using the XTT viability assay. Each data point represents the mean O.D. ± SEM of quadruplicate cultures established from 4 fish (for the 6.25 x 10^3 cell/well group) or 7 fish (for the 2.5 x 10^4 cells/well group). (*) $P < 0.05$, when comparing controls and GMCL-derived CCM treated groups at the same time point and seeded with the same number of leukocytes.

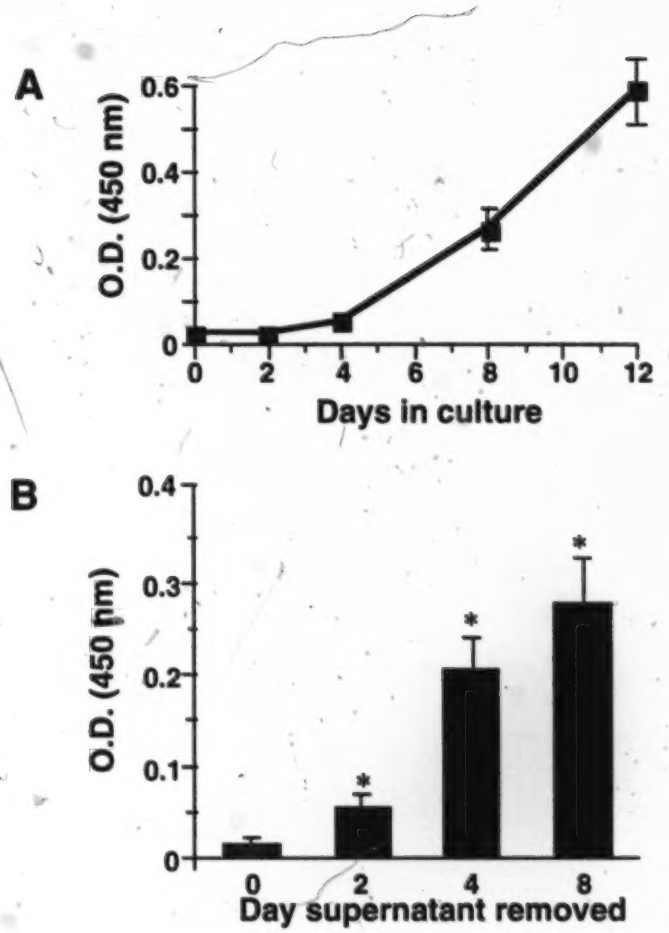

Figure 3.4. Proliferation of $1x10^5$ kidney leukocytes seeded into wells of a 96 well culture plate (A). Supernatants from the cultures in graph (A) were removed at the specified time intervals indicated in (B), and tested for their ability to induce proliferation of cultures seeded with only $1.25x10^4$ kidney leukocytes (B). Cell viability in (A) and (B) were determined using the XTT viability assay (after 12 days of cultivation in (B)). Each data point represents the mean O.D. ± SEM of quadruplicate cultures established from 8 fish. (*) $P < 0.05$, when comparing controls to cultures treated with kidney leukocyte supernatants.

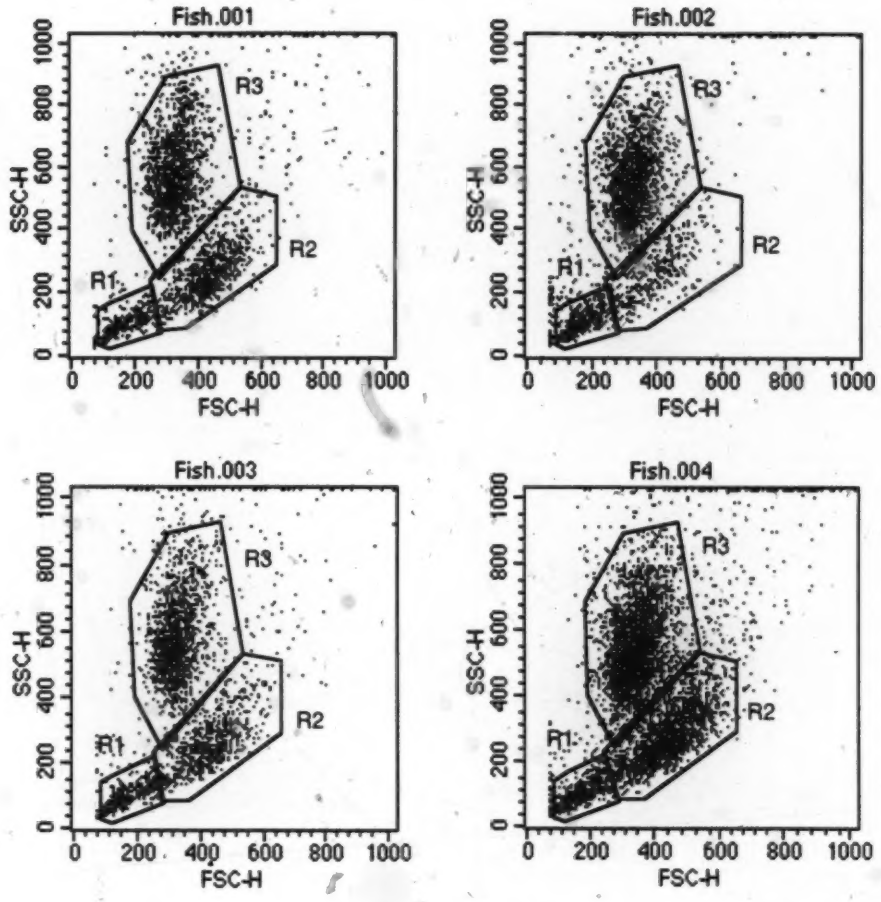

Figure 3.5. Flow cytometric profile of IVDKM cultures obtained from four fish. Cultures were established by seeding $2x10^7$ kidney leukocytes into $75cm^2$ tissue culture flasks and stimulating them to proliferate with 25% (v/v) KL-derived CCM. Flow cytometry was done after 8 days of cultivation. Macrophages segregating to various regions were designated R1, R2 and R3-type macrophages.

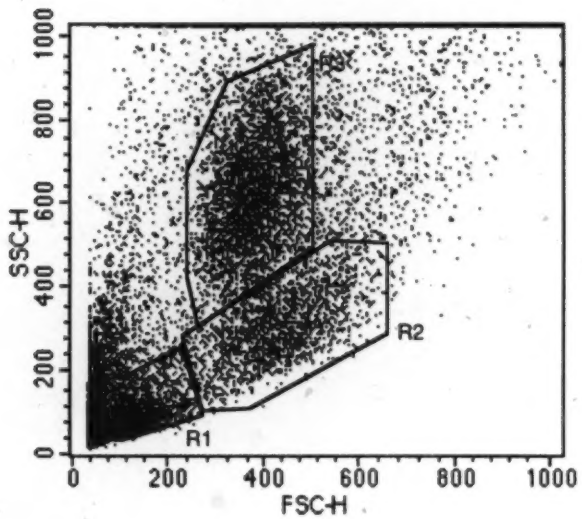

Figure 3.6. Flow cytometric profile of GMCL cultures. The three distinct sub-populations of macrophages seen in IVDKM cultures (see Figure 3.5) are also present in cultures of this long term macrophage cell line.

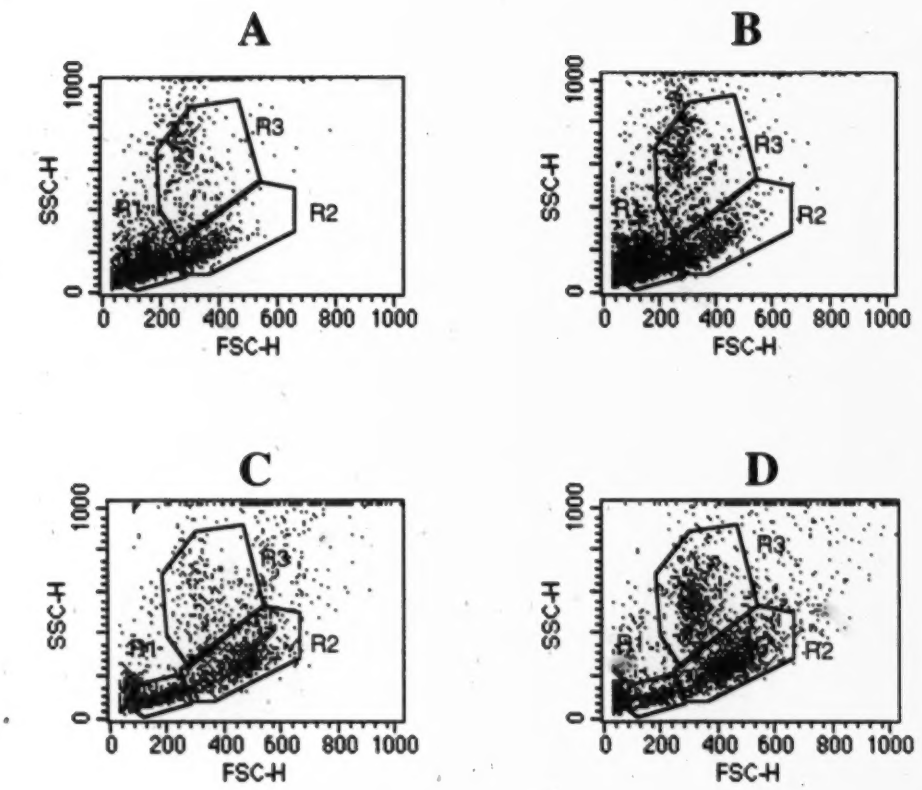

Figure 3.7. Sequence of development of IVDKM as determined by flow cytometry. Kidney leukocytes were seeded into tissue culture flasks and stimulated to proliferate with 25% (v/v) KL-derived CCM. Aliquots were removed from the flasks on days 1 (A), 2 (B), 4 (C), or 8 (D), and the cells analyzed by flow cytometry.

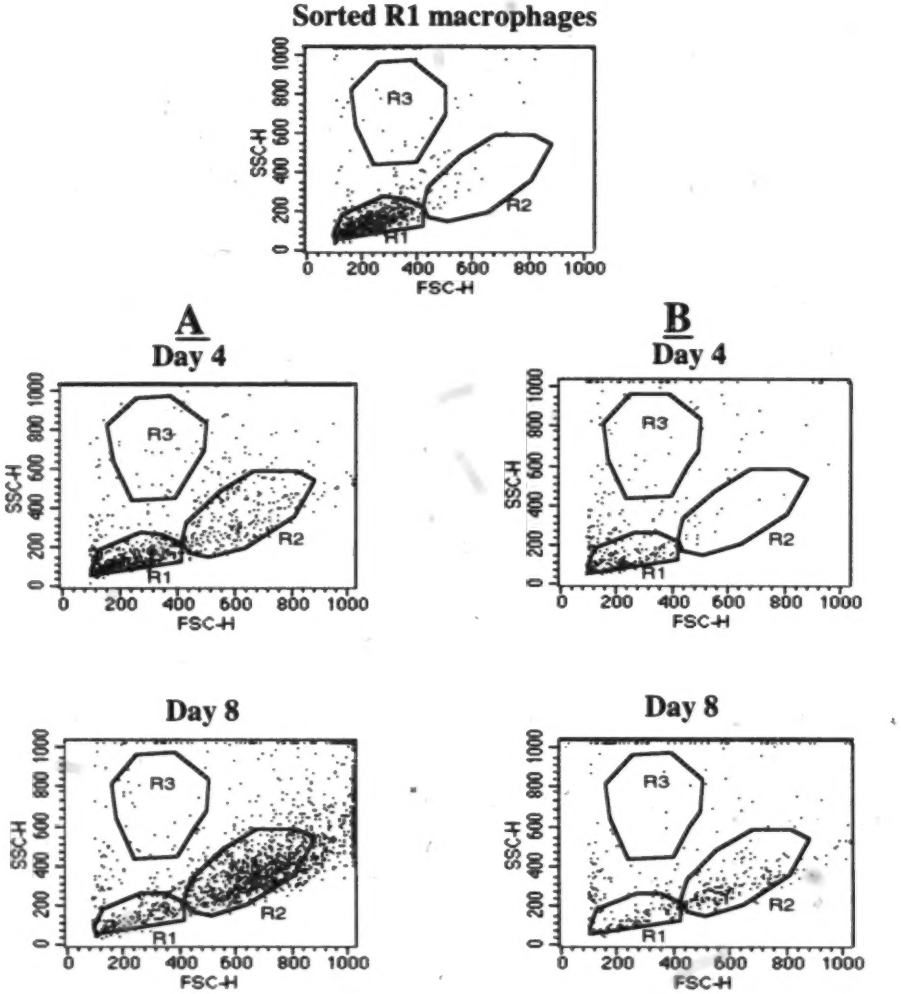

Figure 3.8. Induced differentiation of R1-type macrophages. R1-type macrophages were sorted using flow cytometry and cultured in the presence (panel A) or absence (panel B) of KL-derived CCM. These cells differentiated into R2-type macrophages in both treatment groups. However, differentiation occurs sooner in cultures stimulated with KL-derived CCM, as seen by the appearance of R2-type macrophages after only 4 days of incubation. Unstimulated R1-type macrophages required 8 days of cultivation to differentiate into R2-type macrophages.

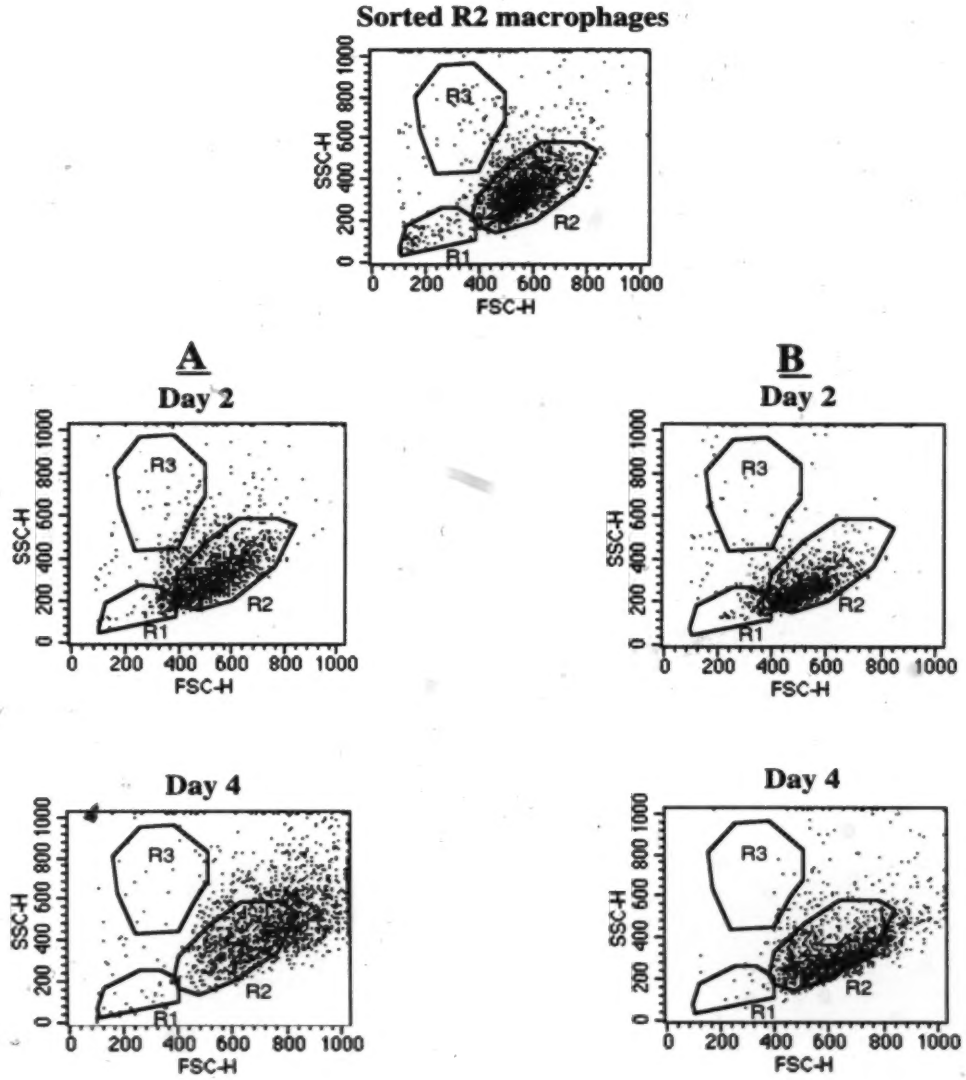

Figure 3.9. Lack of differentiation of R2-type macrophages. R2-type macrophages appear to be a mature sub-population of macrophage in IVDKM cultures, since sorted cells remain in the sort region after 4 days of cultivation, regardless of whether they are cultured in the presence (panel A) or absence (panel B) of KL-derived CCM.

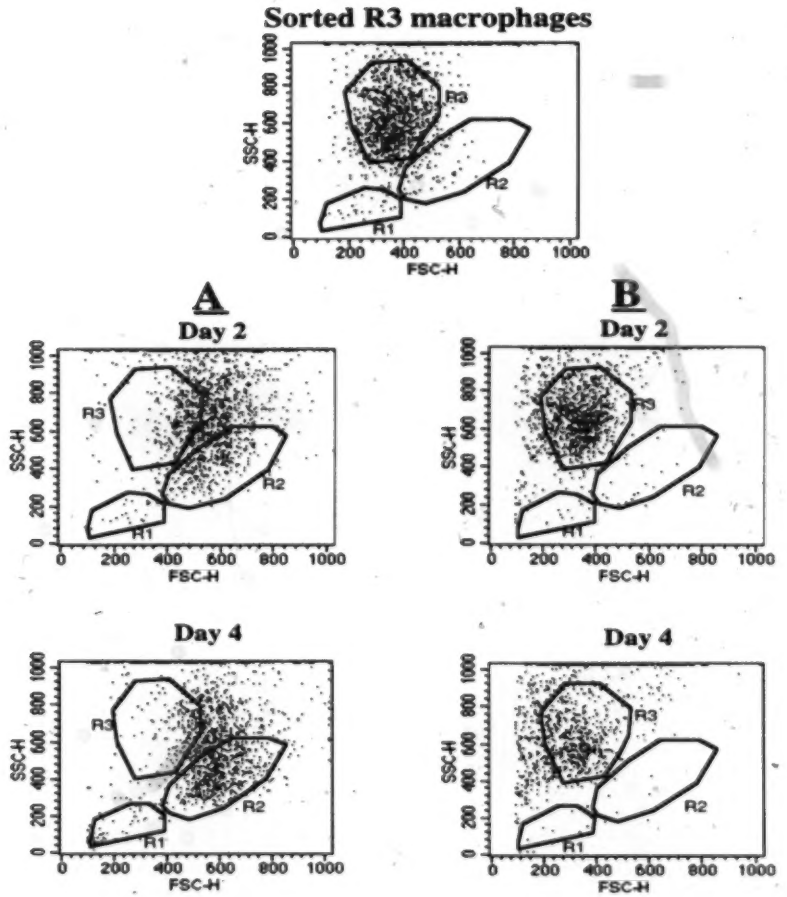

Figure 3.10. Differentiation of R3-type macrophages. R3-type macrophages were sorted using flow cytometry and cultured in the presence (panel A) or absence (panel B) of 25% (v/v) KL-derived CCM. Differentiation of R3-type macrophages into R2-type macrophages occurred only if these cells were cultured in the presence of growth enhancing supernatants. R3-type macrophages are dependent upon the presence of this growth factor(s) for sustained viability, as seen by a left shift in the cytometric profile of these cells by day 4 in panel B (representing increased cellular debris). Death of R3-type macrophages was verified using phase contrast microscopy.

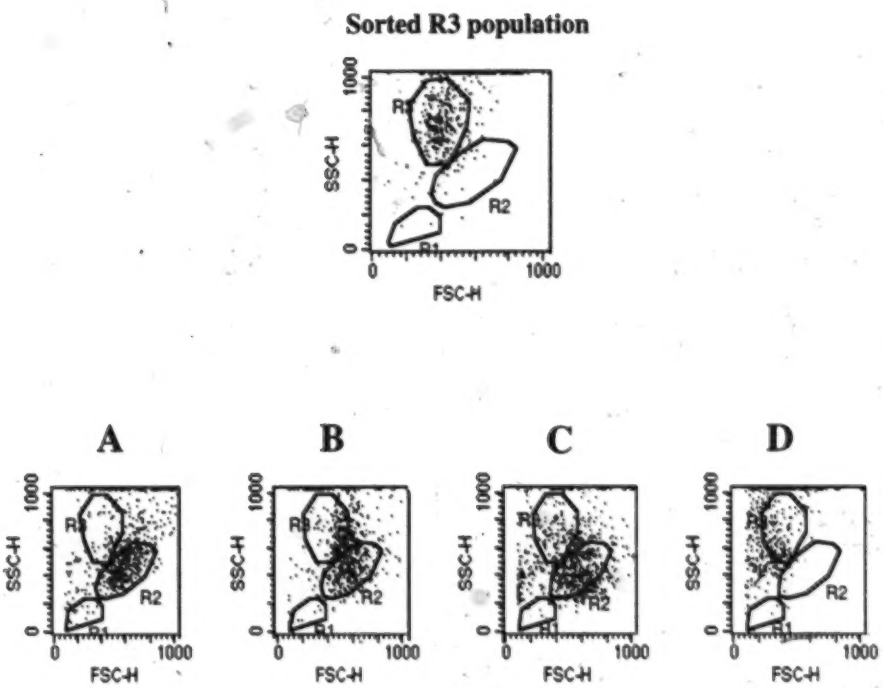

Figure 3.11. Induced differentiation of R3-type macrophages in response to various stimulants. R3-type macrophages were sorted by flow cytometry and cultured in the presence of MAF (1:4 dilution, **A**), KL-derived CCM (25% v/v, **B**), LPS (1 µg/ml, **C**), or medium only (**D**). Macrophages within these cultures were re-analyzed by flow cytometry after 4 days of cultivation.

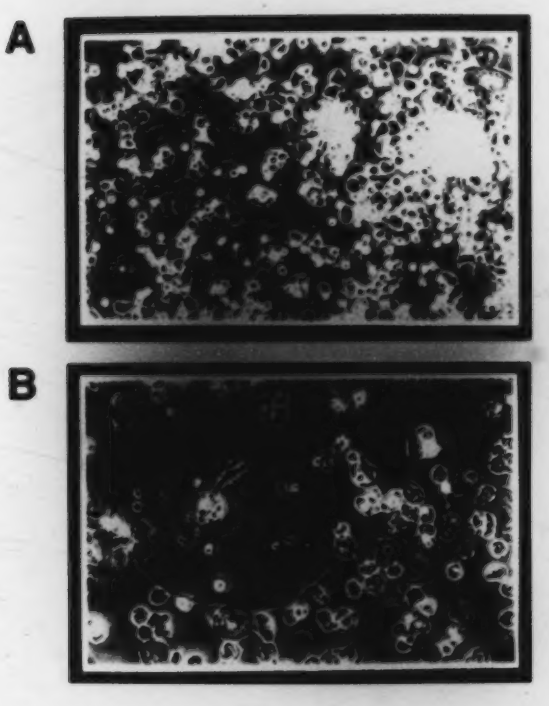

Figure 3.12. Phase contrast photomicrographs of 8 day old IVDKM cultures grown in the presence of KL-derived CCM. Photomicrograph (A) demonstrates the general appearance of actively proliferating cultures containing both adherent and non-adherent cells. In photomicrograph (B), the non-adherent population was removed to demonstrate the characteristic morphology of the adherent cell population. Note the presence of several multinucleated giant cells and the "fried-egg" appearance of the adherent cells.

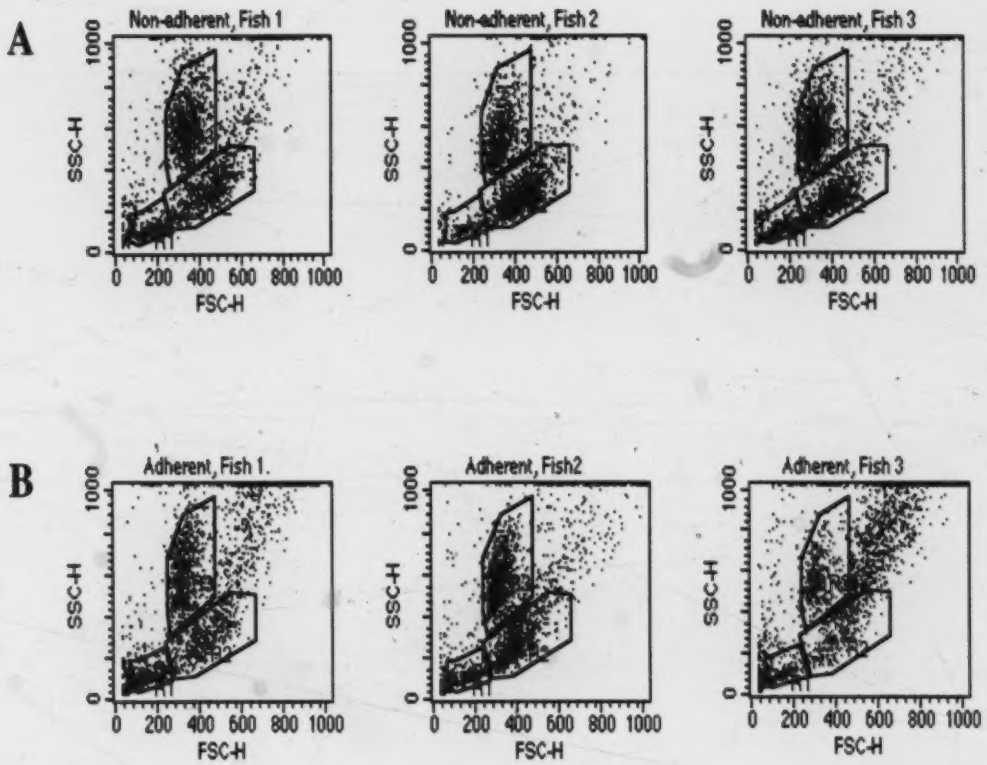

Figure 3.13. Flow cytometric profile of adherent and non-adherent cells in 8 day old IVDKM cultures. Non-adherent cells (panel A) were removed by vigorously shaking IVDKM cultures and pipetting off culture media containing the non-adherent population. Adherent cells were removed by trypsinization (panel B). The data presented above show the flow cytometric profiles of adherent and non-adherent cell populations from 3 fish. Note that the three sub-populations of macrophages (R1-R3) are present in both the adherent and non-adherent cell populations.

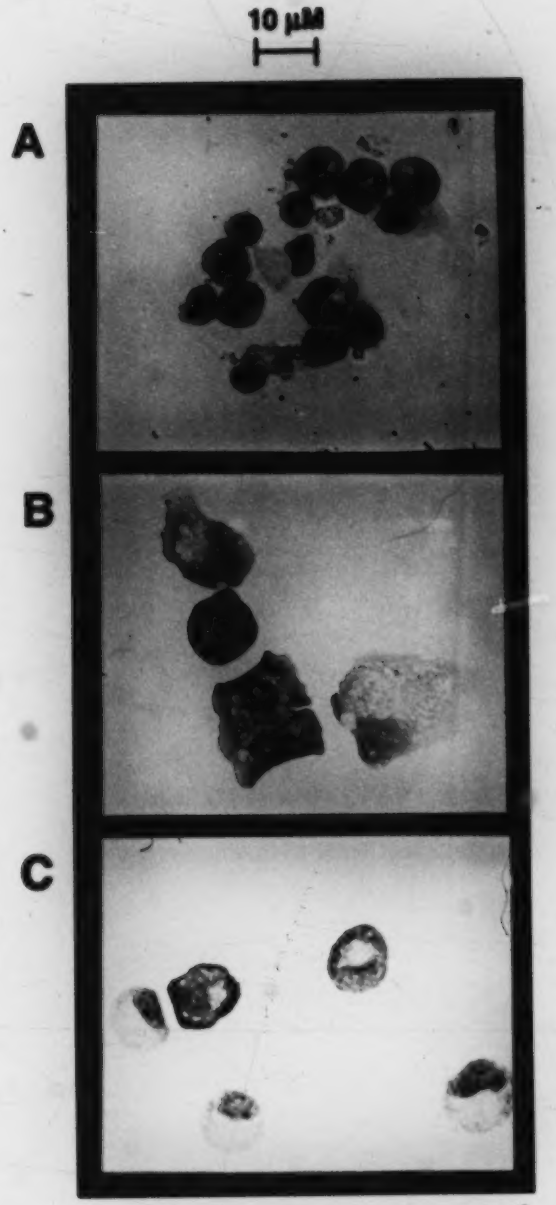

Figure 3.14. Morphological characterization of R1 (A), R2 (B), and R3-type macrophages (C) stained with a Modified Wright's Stain.

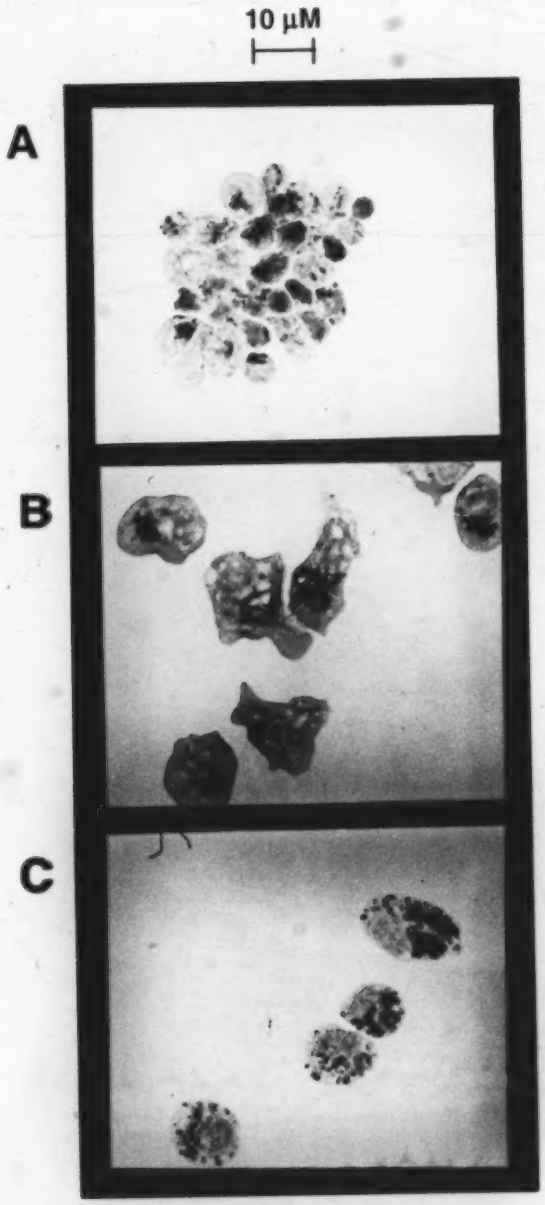

Figure 3.15. Characterization of acid phosphatase activity in R1 (A), R2 (B), and R3-type macrophages (C).

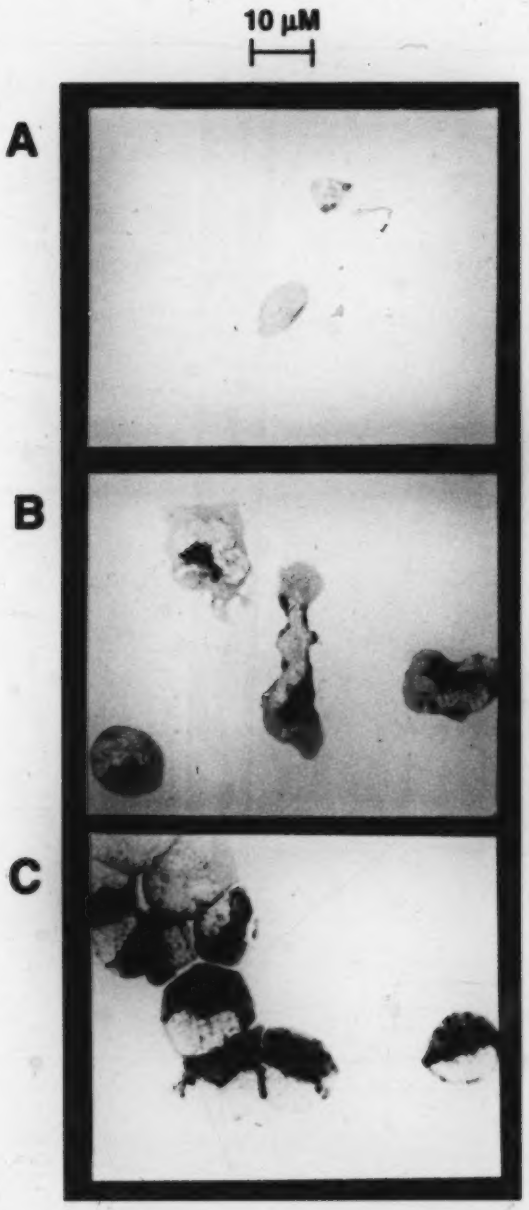

Figure 3.16. Characterization of myeloperoxidase activity in R1 (A), R2 (B), and R3-type macrophages (C).

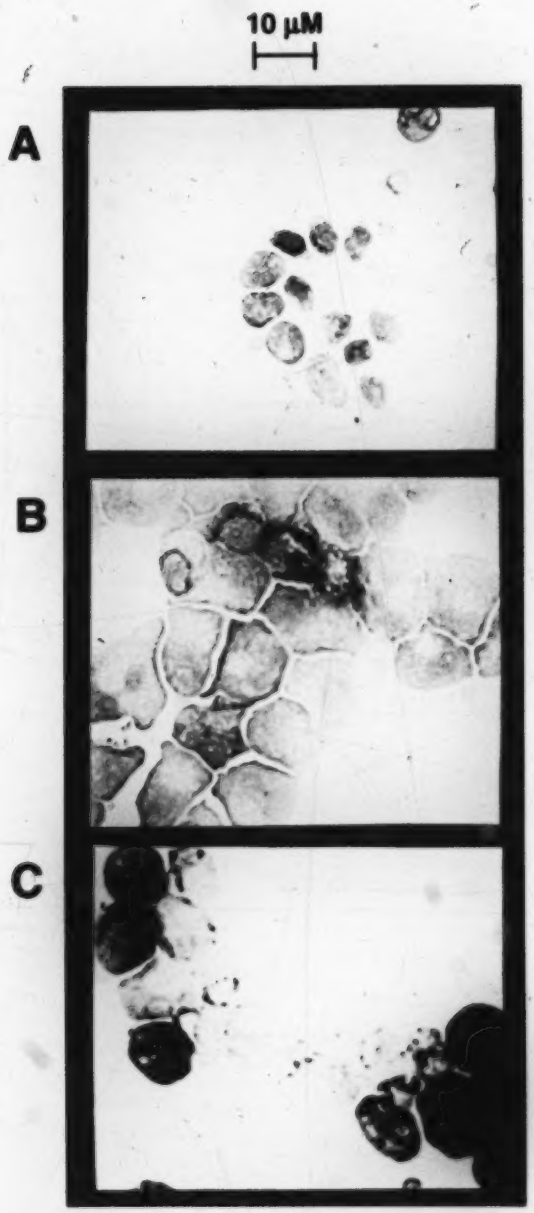

Figure 3.17. Characterization of non-specific esterase activity in R1 (A), R2 (B), and R3-type macrophages (C).

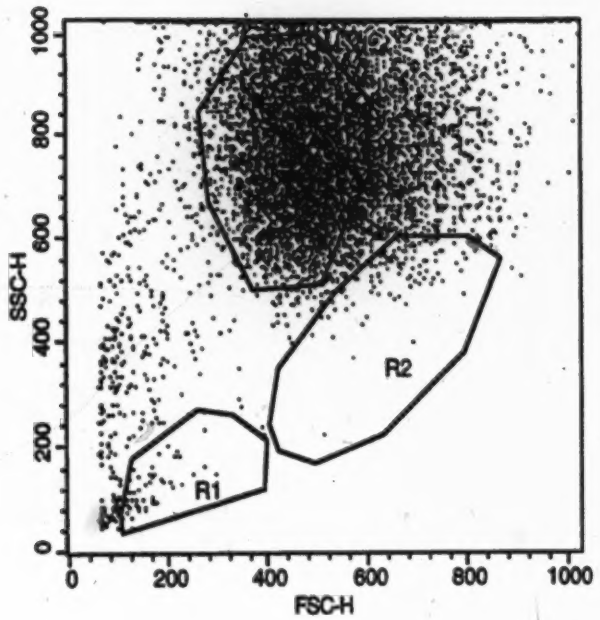

Figure 3.18. Flow cytometric profile of the human monocyte-like cell line, U937, using similar instrument settings as those used for cytometric analysis of goldfish IVDKM.

Figure 3.19. Photomicrograph depicting phagocytosis of the bacterium *Aeromonas salmonicida* by 8 day old IVDKM.

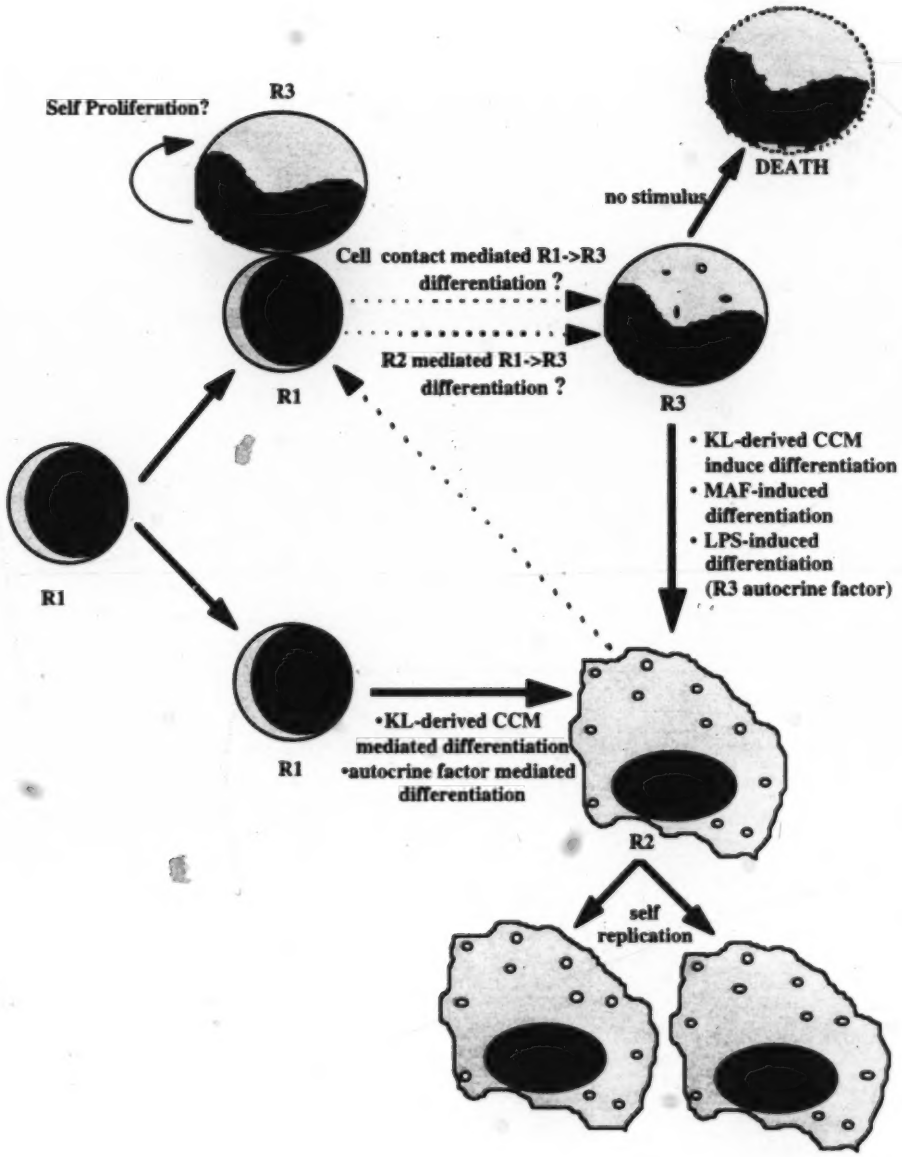

Figure 3.20. A summary of the potential pathways mediating macrophage development in IVDKM cultures.

CHAPTER IV

MECHANISMS OF INDUCTION OF NITRIC OXIDE SYNTHESIS IN GOLDFISH MACROPHAGES [1]

Introduction

Nitric oxide is a pleiotropic molecule whose many functions include intercellular signaling, vasodilation and antimicrobial defense (Nathan, 1992). This reactive nitrogen intermediate is produced through the oxidation of L-arginine (Iyengar et al., 1987; Hibbs et al., 1987a), by an enzyme known as nitric oxide synthase, of which both inducible (iNOS) and constitutive (cNOS) isoforms have been identified in mammals (Stuehr and Griffith, 1992). iNOS has been found in numerous cells including macrophages, neutrophils, eosinophils, hepatocytes, endothelial cells, and fibroblasts (Stuehr and Marletta, 1985; Nussler and Billiar, 1993; Feder and Laskin, 1994; DeVera et al., 1996; Igietseme, 1996; Evans et al., 1996; Wang et al., 1996; Zanardo et al., 1997; del Pozo et al., 1997). Production of nitric oxide by macrophages has received considerable attention, since these cells produce copious amounts of this free-radical molecule in response to inflammatory signals. Induction of nitric oxide production in macrophages occurs as a result of stimulation with cytokines, LPS, and/or parasites (Nathan, 1992). A single stimulus with either IFN-γ or LPS results in low level production of nitric oxide by murine macrophages, whereas co-stimulation with both IFN-γ and LPS results in synergy and the production of large quantities of this cytotoxic molecule (Nathan, 1992; Nussler and Billiar, 1993). Nitric oxide produced by macrophages is thought to play a major role in the cellular

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[1] A version of this chapter has been published: Neumann et al., 1995. *Dev. Comp. Immunol.*, 19: 473-482.

defense against invading pathogens, such as viruses, bacteria, protozoans, helminths, as well as tumors (Nathan and Hibbs, 1991; Nathan, 1992; Table 1.2).

Until recently, no experimental evidence existed which demonstrated nitric oxide production by macrophages of lower vertebrates, such as fish. Schoor and Plum (1994) demonstrated iNOS activity in bacterial challenged catfish kidney homogenates by enzymatic activity. Wang et al. (1995), were the first to provide direct evidence that fish macrophages were capable of producing nitric oxide, by stimulating a goldfish macrophage cell line with bacterial LPS. Most recently, partial cDNA sequences encoding iNOS from both rainbow trout and goldfish macrophages have been reported, with cDNA sequences having approximately 85% homology between these distantly related fish species, and approximately 70% homology with mammalian macrophage iNOS (Laing et al., 1996).

Although fish macrophages were shown to produce nitric oxide in response to stimulation with LPS, no information existed which established a role for cytokines in mediating this antimicrobial response in teleosts. My research demonstrated that MAF, secreted by mitogen stimulated kidney leukocytes, synergizes with LPS for the induction of nitric oxide production in goldfish macrophages (Neumann et al., 1995). Studies done by others have subsequently demonstrated a similar phenomenon (Yin et al., 1997; Mulero and Meseguer, 1998).

This chapter describes the results of experiments that demonstrate the inducibility of nitric oxide production in goldfish macrophages by soluble mediators secreted by goldfish kidney leukocytes. This chapter also describes the kinetics of production and optimal conditions for generating macrophage activating factor(s), as characterized by their ability to induce nitric oxide production by goldfish macrophages. Furthermore, the results described in this chapter define the roles that

pathogens (or pathogen-derived molecules) play in modulating nitric oxide production of cytokine activated goldfish macrophages.

Results

Production of MAF by mitogen-stimulated goldfish kidney leukocytes

MAF was obtained by inducing goldfish kidney leukocytes to secrete these factors into culture medium in response to stimulation with Con A, PMA, and calcium A23187 ionophore. MAF activity in these culture supernatants was assayed for by its ability to induce nitric oxide production in fish macrophages. Since induction of nitric oxide production requires an accessory activating stimulus (such as a pathogen or pathogen-derived molecule), LPS was used as a co-stimulus for activating goldfish macrophages.

Mitogen-stimulated kidney leukocytes secreted a MAF that, when used in conjunction with bacterial LPS, induced goldfish macrophages to produce nitric oxide (Fig. 4.1). PMA and calcium ionophore synergized with Con A for enhanced production of MAF by kidney leukocytes, with the amount of MAF activity present in the culture supernatants dependent on the initial concentration of Con A used to stimulate the leukocytes (Fig. 4.1). Leukocytes stimulated with Con A, or PMA and calcium ionophore alone, produced little or no MAF activity. Maximal MAF activity was observed in leukocyte cultures stimulated with 10-20 µg/ml Con A, 5-20 ng/ml PMA, and 100 ng/ml calcium ionophore. Control supernatants (those supernatants taken immediately from kidney leukocytes stimulated with mitogen), induced similar quantities of nitric oxide as LPS only (3 uM), indicating that residual mitogens were not responsible for activation of the macrophages (Fig 4.2). Moreover,

supernatants obtained from kidney leukocytes not stimulated with mitogen did not enhance nitric oxide production when compared to macrophages stimulated with LPS alone, indicating that production of MAF was dependent on the mitogenic stimulation of kidney leukocytes. Nitrite was not detected in the MAF preparations, indicating that the nitrite produced was synthesized by activated GMCL.

The density at which kidney leukocytes were cultured also affected production of MAF. The greatest nitric oxide-inducing activity was obtained from culture supernatants seeded with larger numbers of kidney leukocytes (i.e. 4-10 $x10^6$ cells/ml, Fig. 4.3). However, because a complete dose response analysis of MAF activity was not done on cultures seeded with 4, 8, or 10 $x10^6$ cells/ml, it is difficult to ascertain whether nitric oxide-inducing activity was greater in cultures seeded with leukocytes exceeding $4x10^6$ cells/ml (Fig 4.3).

Secretion of MAF by mitogen stimulated kidney leukocytes occurred as early as 24 hours after mitogenic stimulation, but maximal nitric oxide-inducing activity was detected in supernatants obtained from 3-4 day old cultures (Fig. 4.3). The temperature at which kidney leukocytes were incubated also affected production of MAF. Supernatants obtained from kidney leukocytes cultured at 30^0C contained more nitric oxide-inducing MAF activity than those cells grown at 10^0C or 20^0C (both comparisons, $P < 0.05$); provided that kidney leukocytes were cultured in medium containing serum (Fig. 4.4).

Based on the results described above, MAF was raised in all subsequent assays by seeding 4×10^6 kidney leukocytes per ml of serum-free medium at 20^0C, and stimulating the leukocytes with 10 µg/ml Con A, 10 ng/ml PMA, and 100 ng/ml Ca^{2+} A23187 ionophore. Serum-free medium was used in order to raise MAF in batch for the eventual purification of the nitric oxide-inducing molecule.

Nitric oxide-inducing activity of MAF samples obtained from individual fish was highly variable. MAF samples from cells cultured under similar conditions had differential abilities to induce nitric oxide production by GMCL (Fig. 4.5). The variability in nitric oxide-inducing potential in these crude cytokine preparations may be explained by the presence of a macrophage deactivator that may affect the overall activation potential of individual MAF preparations (see Chapter VI).

Molecular signals that induce nitric oxide synthesis in goldfish macrophages

Induction of nitric oxide synthesis in goldfish macrophages by MAF and LPS

Treatment of GMCL with MAF or LPS alone resulted in a low level, dose-dependent induction of nitric oxide production (Fig. 4.6). However, co-stimulation of GMCL with both factors resulted in a synergistic induction of this cytotoxic response at all dilutions and concentrations of MAF and LPS tested ($P < 0.05$, Fig. 4.6). Nitric oxide was produced by activated macrophages as early as 24 hrs after stimulation with MAF and LPS, but most nitric oxide was produced between 24-48 hrs (Fig. 4.5). However, nitrite accumulated in the culture medium for up to 96 hrs signifying a prolonged production of this cytotoxic molecule (Fig. 4.5).

Production of nitric oxide, in response to stimulation with MAF and/or LPS, could also be induced in IVDKM (Fig. 4.7), suggesting that these signals are important physiological stimuli *in vivo* for activating teleost macrophages to produce nitric oxide. Although IVDKM produced nitric oxide when stimulated with MAF and/or LPS, R2-type macrophages appeared to be the cell type responsible for its production. R2-type macrophages demonstrated a classical macrophage response; i.e.

little or no production of nitric oxide when stimulated with MAF or LPS, but copious production when co-stimulated with both factors (Fig. 4.8). Interestingly, R3-type macrophages produced only small amounts of nitric oxide in response to stimulation with MAF or LPS, or combinations of these factors (Fig. 4.8 and Table 6.1).

Induction of nitric oxide synthesis in goldfish macrophages by pathogens

Pathogens can act as synergistic signals for induction of nitric oxide production in macrophages. LPS represents the stimulating factor induced by Gram negative bacteria, organisms known to be a potent stimulators of macrophage cytotoxic responses. I therefore examined whether LPS-deficient pathogens were capable of inducing nitric oxide production in fish macrophages. The pathogens examined were: 1) *L. major*, an obligate intracellular protozoan pathogen of mammalian macrophages, previously shown to be capable of infecting goldfish macrophages (Wang et al., 1995); and 2) *Mycobacterium chelonei* , a Gram positive bacterial fish pathogen that causes piscine tuberculosis.

Induction of nitric oxide production by Leishmania major

Goldfish macrophages produced nitric oxide in response to intracellular infection with the protozoan pathogen, *L. major*. The amount of nitric oxide produced was dependent on the number of parasites initially used to infect macrophages (Fig. 4.9). In experiments where nitric oxide was determined after 96 hrs of infection, MAF was shown to significantly enhance production of nitric oxide by infected goldfish macrophages (Fig. 4.9), providing that relatively few parasites were used to infect macrophages (i.e. 1:1 parasite to macrophage infection ratio). In IVDKM cultures infected at 5:1 or 10:1 parasite to macrophage

ratios, MAF significantly inhibited ($P<0.05$) nitric oxide production (Fig 4.9).

Interestingly, MAF also augmented nitric oxide production of infected macrophages early after infection (Fig. 4.10). Macrophages infected with *L. major* and co-stimulated with MAF began producing detectable levels of nitrite in supernatants by 24 hrs (Fig 4.10). In this particular experiment, *L. major*- infected macrophages co-stimulated with MAF produced significantly more nitric oxide than infected macrophages at all time points examined. However, after 96 hours the quantities of nitric oxide produced by infected macrophages were almost comparable to those co-stimulated with MAF (Fig. 4.10).

Unlike goldfish macrophages, murine macrophages did not produce nitric oxide in response to infection with *L. major* (Table 4.1). *L. major*-infected murine macrophages required IFN-γ as an accessory stimulus for induction of nitric oxide production (Table 4.1).

The production of nitric oxide by goldfish macrophages was not simply a result of phagocytosis of *L. major* , since phagocytosis of latex beads did not stimulate nitric oxide production in IVDKM (Table 4.2). Thus, induction of nitric oxide production by goldfish macrophages infected with *L. major* must be dependent upon receptor-mediated events involving the recognition of pathogen-derived molecules.

Induction of nitric oxide production by Mycobacterium chelonei

Goldfish macrophages also produced nitric oxide in response to infection with the Gram positive, bacterial fish pathogen, *M. chelonei* (Fig. 4.11). Production of nitric oxide by infected macrophages was significantly enhanced when macrophages were co-stimulated with MAF (Fig. 4.11).

Discussion

Effective elimination of infectious pathogens by macrophages is dependent upon two important signals that must be perceived by macrophages; the recognition of pathogen-derived molecules and the stimulation of macrophages with activating cytokines. These two fundamentally important signals initiate the induction of macrophage antimicrobial effector mechanisms designed to destroy the invasive pathogens. Nitric oxide produced by activated macrophages is a critical effector molecule involved in the destruction of many pathogenic microorganisms (Table 1.4).

My results demonstrate that host-derived soluble mediators (i.e. MAF) play an important role in the induction of nitric oxide production by goldfish macrophages. Since no purified or recombinant cytokines are currently available in teleosts, experiments attempting to define the importance of these molecules in modulating macrophage activation in fish depends on the generation of crude cytokine preparations. MAF was produced from goldfish kidney leukocytes stimulated to secrete these factors into culture medium in response to stimulation with mitogens. The presence of MAF within crude cytokine preparations was assayed by the ability of individual MAF preparations to induce goldfish macrophages to produce nitric oxide. Production of MAF by kidney leukocytes was influenced by several factors including; mitogenic stimulation, kidney leukocyte density, the presence of serum, and temperature.

Kidney leukocytes stimulated with Con A or PMA and calcium ionophore produced little or no detectable MAF activity. However, stimulation of leukocytes with a combination of these mitogens resulted in a synergistic production of MAF activity. This increased activity was dependent upon the concentration of Con A. Optimal MAF production

appeared to occur in cultures stimulated with 10 µg/ml Con A, 10 ng/ml PMA and 100 ng/ml calcium ionophore. These results do not determine whether PMA and calcium ionophore synergize with Con A for increasing production of a MAF by existing leukocytes, or whether the combination of these mitogens induces proliferation of existing populations of cells responsible for the production of MAF.

The production of MAF by kidney leukocytes was also dependent upon the incubation temperature and the presence of serum in mitogen stimulated leukocyte cultures. Supernatants obtained from leukocytes cultured at 30°C in the presence of serum contained higher MAF activity than leukocytes raised at lower temperatures in the presence or absence of serum.

Based on the experiments presented in this chapter, it is difficult to determine whether treatment conditions such as mitogenic concentration, temperature, presence/absence of serum, or cell seeding density, actually increase production of a specific MAF by kidney leukocytes, since complete dose response analyses were not conducted on all MAF samples. Clearly, the production of a MAF by kidney leukocytes is highly variable, and under similar culture conditions MAF preparations can vary in their ability to induce nitric oxide production in goldfish macrophages. Moreover, the increase in MAF activity observed in some cultures under certain treatment conditions cannot be interpreted as increased production of a specific MAF molecule. The increased activity may simply reflect a balance between macrophage activating factors and macrophage deactivating factors present in crude cytokine preparations (Chapter VI). Thus, cytokine preparations having little or no deactivator present may display high nitric oxide inducing activity, although actual quantities of the activating MAF may be less.

My results do support the findings of Hardie *et al.* (1994) who showed that production of MAF by rainbow trout kidney leukocytes was

influenced by incubation temperature. Several studies have shown the temperature dependence of immune responses in fish, such as cell proliferation, antigen presentation, phagocytosis and antibody production (Bly and Clem, 1992). Interestingly, fish often show a behavioral response to infection, by moving into warmer waters during bouts of infection (Cabanac and Laberge, 1998).

Both GMCL and IVDKM treated with either MAF or LPS alone induced relatively little nitric oxide production, as compared to the synergistic induction after co-stimulation with both factors. The amount of nitric oxide produced by activated macrophages was dose dependent for both stimulants. Recently, two other groups, working with catfish (*Clarias gariepinus*) and gilthead seabream (*Sparus aurata*), also demonstrated nitric oxide production by macrophages stimulated with MAF and LPS (Yin et al., 1997; Mulero and Meseguer, 1998). Yin et al., (1997) have also demonstrated that nitric oxide produced by catfish macrophages plays an important role in the bactericidal activity of these cells against *Aeromonas hydrophila*.

Interestingly, R2-type macrophages appear to be the sub-population responsible for the nitric oxide produced by IVDKM and GMCL cultures. R2-type macrophages demonstrated a prototypical macrophage nitric oxide response, producing little or no nitric oxide in response to stimulation with MAF or LPS, but producing copious amounts in response to co-stimulation with both factors. R3-type macrophages produced very little nitric oxide in response to stimulation with these factors. Although R3-type macrophages morphologically and cytochemically resemble monocytes (Chapter III), their inability to produce nitric oxide, in response to stimulation with cytokines and LPS, is similar to what is observed in human monocytes and caprine monocyte-derived macrophages (Schneemann et al., 1993; Adler et al., 1996). The inability of human, caprine, and goldfish monocytes to

produce nitric oxide is in contrast to bovine monocyte-derived macrophages or murine macrophages, which produce copious amounts of nitric oxide in response to stimulation with cytokines and LPS (Stuehr and Marletta, 1985; Adler et al., 1996). Nevertheless, at least two reports demonstrated that human monocytes matured *in vitro* acquire the capacity to produce nitric oxide in response to stimulation with cytokines and LPS (Martin and Edwards, 1993; Bose and Farnia, 1995). Therefore, the differentiation of R3-type goldfish macrophages into the more mature R2-type macrophages (Chapter III) may be a pre-requisite for acquiring the capacity to generate nitric oxide.

Unique to fish macrophages is their ability to produce nitric oxide in response to intracellular infection. Goldfish macrophages infected with *L. major* produced large amounts of nitric oxide, the production of which could be enhanced by co-stimulation with MAF. MAF enhanced nitric oxide production during the first 72 hrs of infection. However, macrophages infected with *L. major* only produced similar levels of nitric oxide as those co-stimulated with MAF by 96 hrs. Thus, MAF may play an important role in teleost macrophage cytotoxicity by augmenting nitric oxide responses in early stages of infection, and synergistically inducing this response when few pathogens gain access to host tissues.

These results are in contrast to those obtained using murine macrophages. Murine macrophages (J774A.1 cell line), did not produce nitric oxide in response to infection with *L. major*, but required IFN-γ as a co-stimulus for induction of this cytotoxic response. The requirement of *L. major* -infected murine macrophages to be co-stimulated with IFN-γ, for induction of nitric oxide production is consistent with that observed by others (Green et al., 1990; Green et al., 1991; Guy and Belosevic, 1993).

The ability of goldfish macrophages to produce nitric oxide in response to intracellular infection is not a primitive cellular response

induced by phagocytosis. Phagocytosis of latex beads by goldfish macrophages did not induce nitric oxide production. Thus, the induction of this antimicrobial response in goldfish macrophages by pathogens depends on receptor-mediated signaling involving the recognition of pathogen-derived molecules. The induction of nitric oxide by infected goldfish macrophages, however, may represent a more primitive form of regulation of this antimicrobial response. In mammals, nitric oxide production appears to become more tightly regulated, and is dependent upon stimulation by both cytokines and pathogen-derived molecules. My results indicate that the cytokine-induced regulation of nitric oxide production by teleost macrophages may be similar to that of mammals under certain circumstances, in that stimulation of macrophages with cytokines and pathogen-derived molecules results in the synergistic induction of nitric oxide production in both teleosts and mammals.

It is possible that a TNFα-like molecule secreted by LPS-stimulated macrophages provides the necessary synergistic signal along with MAF for induction of nitric oxide production. Recently, Hardie et al (1994) demonstrated that human TNF-α augments respiratory burst activity in rainbow trout macrophages primed with MAF. The presence of TNF-α, therefore, may be critical for induction of nitric oxide production by teleost macrophages, similar to that observed for murine macrophages (Nacy et al., 1991; Beyaert and Fiers, 1994).

Several reports have shown that MAF secreted by rainbow trout T-cells induces respiratory burst and microbicidal activity in trout macrophages (Secombes, 1987; Chung and Secombes, 1987; Graham and Secombes, 1990 a,b; Sharp and Secombes, 1993), and that the MAF is both heat and acid labile (Graham and Secombes, 1990b). To date, the molecular characteristics of MAF produced by mitogen-stimulated fish leukocytes remain to be elucidated, but it has been suggested that the

molecule may be similar to mammalian IFNγ (Graham and Secombes, 1990a). IFNγ, is a lymphokine known to induce reactive oxygen and nitrogen intermediates in mammalian macrophages and is also heat and acid labile (Farrar and Schreiber, 1993; Stuehr and Marletta, 1987; Murray, 1988). It is likely that fish possess an IFN-γ -like molecule, since the induction of such a potent killer phenotype from a normally quiescent macrophage would require that its activation be highly regulated, as is the case in mammals (Nacy and Meltzer, 1991). Macrophage activation in mammals is regulated by a number of different cytokines; IFN-γ, IL-1, IL-2, GM-CSF and TNFα, that promote macrophage activation (Nacy and Meltzer, 1991; Celada and Nathan, 1994), and TGF-β, IL-4 and IL-10 that promote macrophage deactivation (Bogdan and Nathan, 1991; Bogdan et al., 1991; Bogdan et al., 1994). Although there is growing evidence for factors which promote activation in teleost macrophages, experimental evidence for deactivators of teleost macrophages have only recently been investigated. Jang et al. (1994), have demonstrated downregulation of respiratory burst activity in rainbow trout macrophages following stimulation with bovine TGF-β.

The biochemical pathways leading to nitric oxide production in fish macrophages appear to be very similar to those found in mammalian macrophages. Nitric oxide production in goldfish macrophages is dependent upon L-arginine, as demonstrated by the inhibition of nitric oxide production using the L-arginine analogs, N^{G}MMLA and amino guanidine (Wang et al., 1995; Neumann et al., 1995). The presence of this enzyme has been confirmed at the nucleotide sequence level by Hardie *et al.* (1994) and Laing et al. (1996), who have recently reported partial cDNA sequences for rainbow trout and goldfish iNOS, respectively. The nucleotide sequence homology of this enzyme in these two diverse teleost species is 85% homologous, and is

approximately 70% homologous to published mammalian iNOS sequences (ranging from 67-75%, Laing et al., 1996).

The relative similarities in the mechanisms of induction, and the biochemical pathways leading to nitric oxide production between mammalian and fish macrophages suggest that the induction of this antimicrobial effector response in macrophages is highly conserved.

Table 4.1. Comparison of nitric oxide production between a goldfish and mouse macrophage cell line in response to infection with *Leishmania major*[a].

Macrophage Cell line	Cytokine stimulus	Infection ratio (parasite : macrophage)	µM Nitrite (± SEM)
GMCL (goldfish)	none	1:1	3.5 ± 0.8
		5:1	33.4 ± 1.3
		10:1	35.1 ± 6.7
	MAF (1:4)	1:1	14.5 ± 1.3
		5:1	45.4 ± 4.8
		10:1	54.1 ± 10.6[b]
J774A.1 (mouse)	none	1:1	0.2 ± 0.1
		5:1	0.4 ± 0.4
		10:1	0.4 ± 0.4
	IFN-γ (25 U/ml)	1:1	53.6 ± 2.3[b]
		5:1	78.6 ± 7.8[b]
		10:1	94.2 ± 6.3[b]

[a] Macrophage were seeded into polypropylene tubes ($5x10^5$ cells/tube) and infected with *L. major* at the infection ratios listed. Nitrite content was determined using the Griess reagents after 72 hrs of incubation. Data is presented as the mean ± SEM of triplicate cultures and is representative of a single experiment.

[b] The value listed in the cytokine-treated group is significantly different than the value obtained in parallel cultures stimulated with parasite only ($P < 0.05$).

Table 4.2. Induction of nitric oxide production in IVDKM by phagocytosis of *L. major* or latex beads.

Phagocytic target	target : macrophage ratio	μM nitrite (± SEM)
L. major	1 : 1	4.4 ± 0.14
	5 : 1	6.8 ± 0.18
	10 : 1	7.9 ± 0.09
	20 : 1	7.8 ± 0.06
latex beads	10 : 1	-1.0 ± 0.09
	20 : 1	-1.1 ± 0.07
	40 : 1	-0.95 ± 0.04
	80 : 1	-0.88 ± 0.06

[a] Macrophage were seeded into a half area 96 well culture plate ($5x10^4$cells/well) and allowed to phagocytose *L. major* or latex beads at the infection ratios listed. Nitrite content was determined using the Griess reagents after 48 hrs of incubation. Data is presented as the mean ± SEM of triplicate cultures.

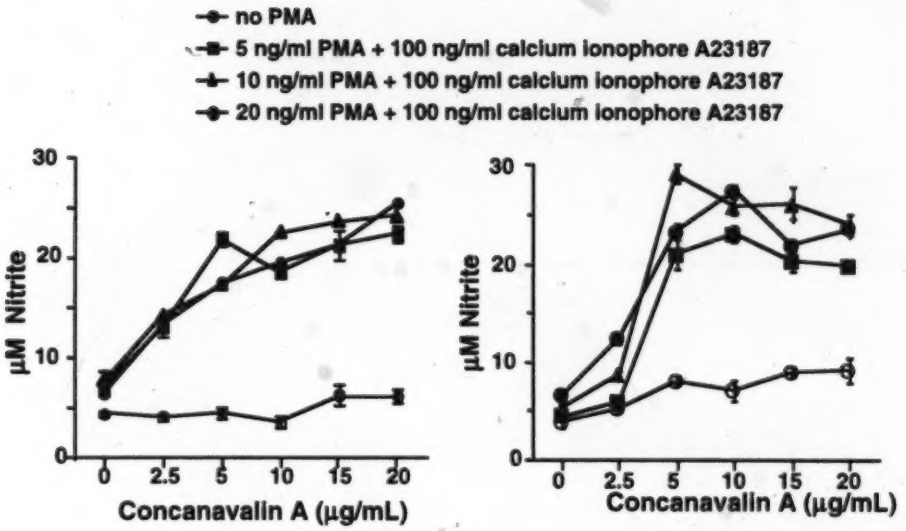

Figure 4.1. Effect of mitogens on MAF production by goldfish kidney leukocytes, as assayed by the ability of MAF to induce nitric oxide production in GMCL. Kidney leukocytes were seeded at densities of $2x10^6$ cells/ml in 24 well tissue culture plates, and pulsed with mitogens (Con A, PMA, and calcium ionophore) for 6 hrs at the specified concentrations. All cultures stimulated with PMA were also stimulated with 100 ng/ml calcium ionophore A23187. Kidney leukocytes were subsequently washed to remove mitogens, and the cells cultured for an additional 72 hrs. Supernatants were removed from the cultures and tested for their ability to induce nitric oxide production in GMCL. GMCL were seeded in 96 well culture plates ($2x10^5$ cells/well) and stimulated with a 1:4 dilution of MAF containing supernatants and 5µg/ml LPS. Nitrite production was determined 72 hrs later using the Griess reagents. Each data point represents the mean ± SEM of triplicate cultures. The data from the two experiments shown (A and B) are representative of five that were performed.

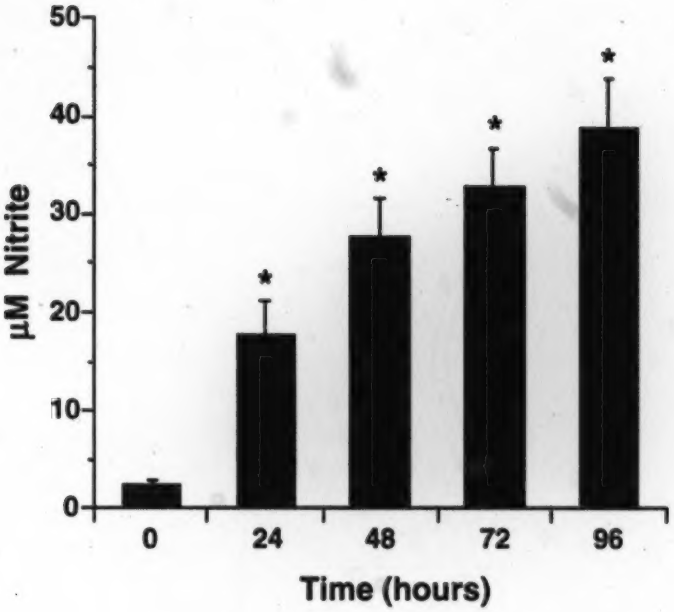

Figure 4.2. Time course of MAF production by goldfish kidney leukocytes, as determined by the ability of MAF to induce nitric oxide production in GMCL. Goldfish kidney leukocytes were seeded at densities of $4x10^6$ cells/ml in 24 well tissue culture plates and stimulated with mitogens (10 µg/ml ConA, 10 ng/ml PMA, and 100 ng/ml calcium ionophore A23187) to secrete MAF. MAF containing supernatants were collected after the specified time periods. GMCL ($2x10^5$ cells/well) were stimulated with a 1:4 dilution of these MAF preparations and 5 µg/ml LPS. Nitrite produced by activated macrophages was determined after 72 hrs of cultivation. MAF preparations produced from 4 fish were tested at each time point and the data are the mean ± SEM. The data are from a representative experiment of two that were performed. (*) $P < 0.05$ comparing the specified time periods to controls (0 hrs).

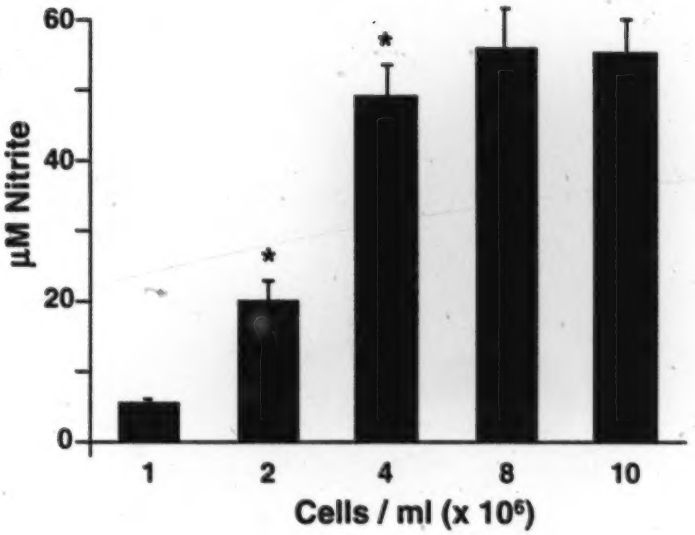

Figure 4.3. Effect of cell density on production of MAF by mitogen stimulated kidney leukocytes. Kidney leukocytes were seeded into wells of a 24 well culture plate at the specified densities and stimulated with mitogens (10 µg/ml ConA, 10 ng/ml PMA, and 100 ng/ml calcium ionophore A23187) to secrete MAF. MAF containing supernatants were collected after 72 hrs. MAF activity was determined by stimulating GMCL with these MAF containing supernatants (1:4 dilution) and LPS (5 µg/ml). Nitrite production of activated macrophages was determined 72 hrs later using the Griess reagents. Each data point represents the mean (± SEM) nitrite production induced by 8 different MAF preparations. (*) $P < 0.05$ comparing nitric oxide inducing capability of the specified MAF to the MAF raised using the previous lower cell concentration.

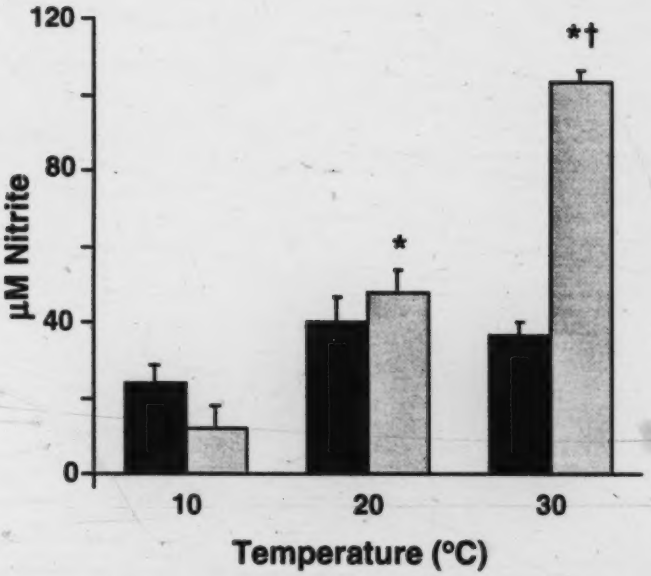

Figure 4.4. Effect of incubation temperature and serum on the ability of mitogen stimulated kidney leukocytes to produce MAF, as determined by the their ability to induce nitric oxide production in GMCL. Kidney leukocytes were cultured at densities of $4x10^6$ cells/ml in 24 well tissue culture plates and stimulated with mitogens (10 µg/ml ConA, 10 ng/ml PMA, and 100 ng/ml calcium ionophore A23187) to secrete MAF. Leukocytes were cultured with (grey) or without serum (black) at the specified temperatures. MAF-containing supernatants were collected after 72 hrs. MAF activity was determined by stimulating GMCL with a 1:4 dilution of MAF supernatants and 5 µg/ml LPS. Nitrite produced by activated macrophages was determined 72 hrs later using the Griess reagents. Four MAF supernatants obtained from 4 fish were tested (each in triplicate) at each time point and represent the mean ± SEM. The data are from a representative experiment out of two that were performed. (*)$P < 0.05$ when comparing adjacent treatment groups with serum (i.e. effect of temperature). (†) $P < 0.05$ when comparing the presence and absence of serum in treatment groups raised at the same temperature.

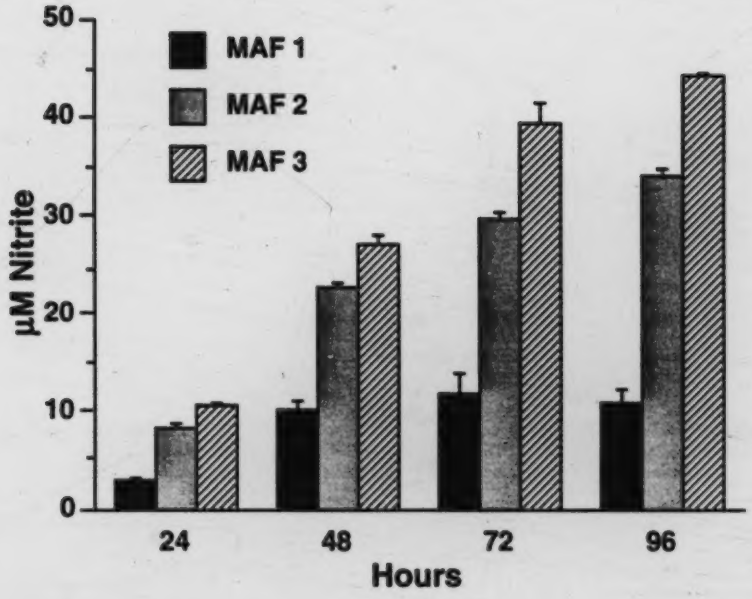

Figure 4.5. Variability in the nitric oxide-inducing potential of different MAF preparations. GMCL ($2x10^5$ cells/well) were co-stimulated with individual MAF samples (1:4 dilution) and LPS (5 µg/ml), and nitrite produced by activated macrophages determined at each of the specified time points. Note that in addition to the weak nitric oxide-inducing potential of MAF 1, macrophages stimulated with this sample reached maximal nitrite production after 48 hrs, compared to 96 hrs in the other MAF samples. MAF samples were raised under similar conditions, but were obtained from 3 fish. Each data point represents the mean ± SEM of quadruplicate cultures.

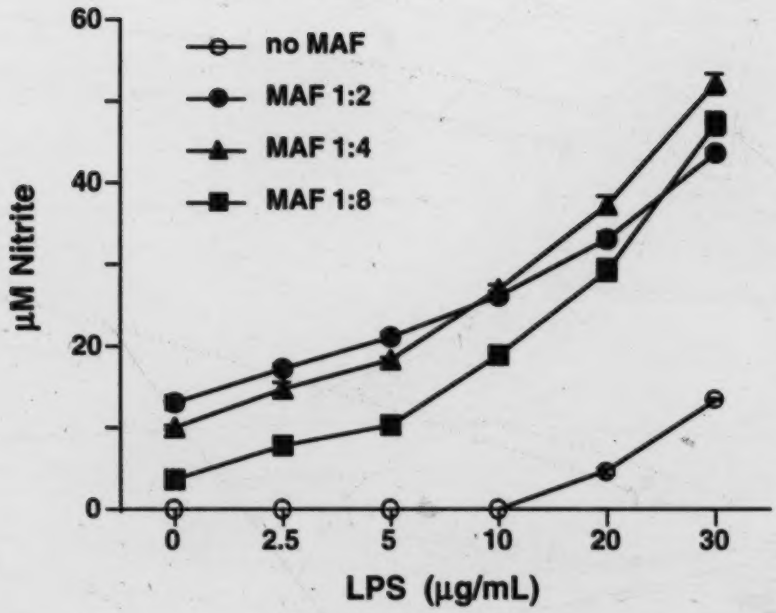

Figure 4.6. The induction of nitric oxide production by GMCL stimulated with MAF and/or LPS. GMCL were seeded in wells of a 96 well culture plate ($2x10^5$ cells/well) and stimulated with the specified concentrations of MAF and/or LPS, and nitrite concentration determined after 72 hrs of cultivation using the Griess reagents. Each data point represents the mean ± SEM from triplicate cultures. The data are from a representative experiment of two that were performed.

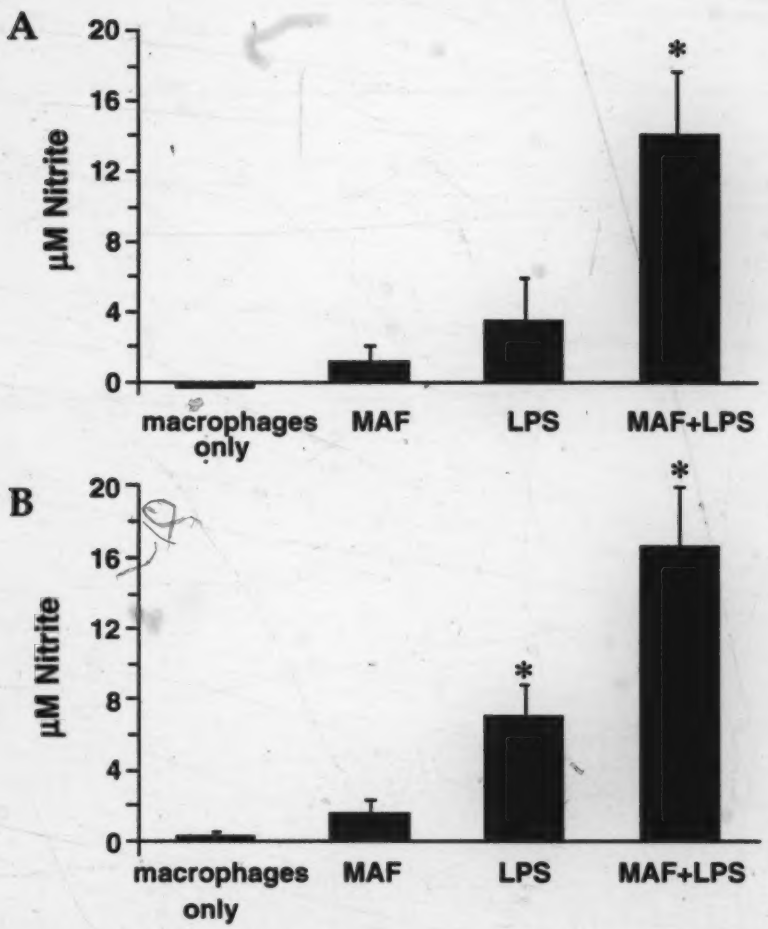

Figure 4.7. Induction of nitric oxide production in IVDKM cultures. IVDKM cultures were established using GMCL-derived CCM (A) or KL-derived CCM (B), and tested for their ability to produce nitric oxide in response to stimulation with MAF (1:4 dilution) or LPS (5 µg/ml in "A", and 1 µg/ml in "B"). Cell numbers used in the experiments were $2x10^5$ cells/well (96 well culture plate) for (A), and $5x10^4$ cells/well (1/2 area 96 well culture plate) for (B). Nitrite content was determined 72 hrs later using the Griess reagents. Each data point represents the mean ± SEM of IVDKM cultures established from seven fish in (A) or eight fish in (B). (*) $P < 0.05$ when comparing treated groups to control.

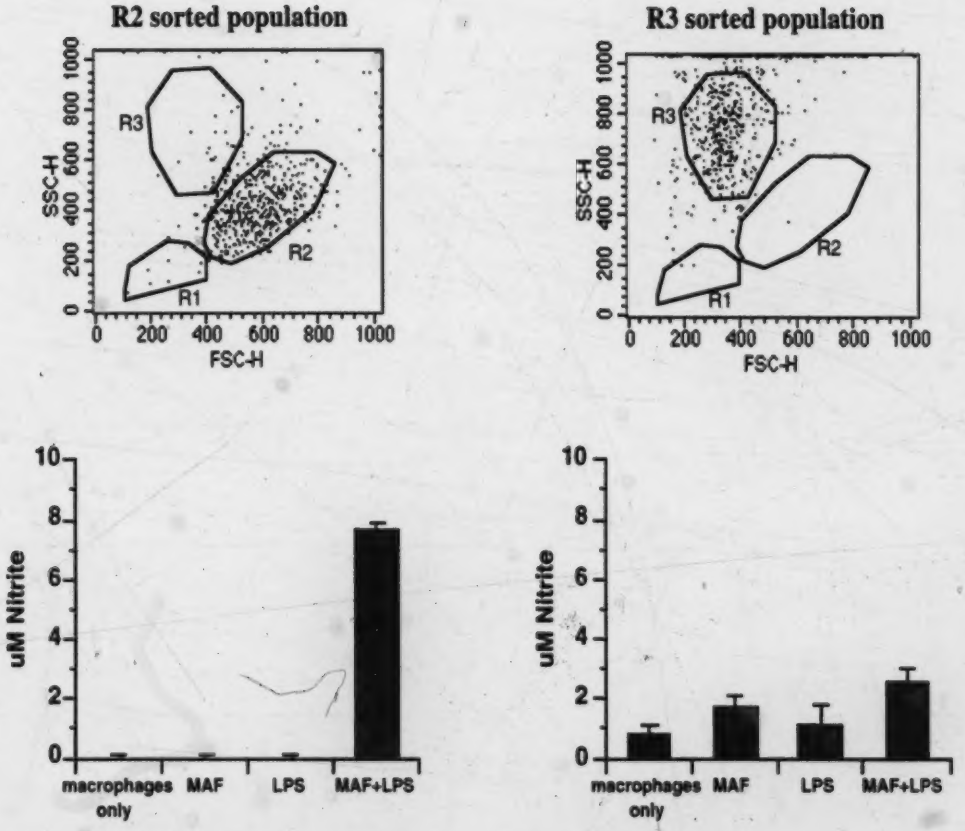

Figure 4.8. Production of nitric oxide by R2 and R3-type macrophages in IVDKM cultures. R2 and R3-type macrophages were sorted using flow cytometry and seeded into wells of 1/2 area 96 well culture plates ($2.5x10^4$ cells/well) and stimulated to produce nitric oxide in response to stimulation with MAF (1:4 dilution) and LPS (1 μg/ml). Nitrite produced by activated macrophages was determined 72 hrs later. Each data point represents the mean ± SEM of triplicate cultures. The data presented are from a representative experiment of two that were performed.

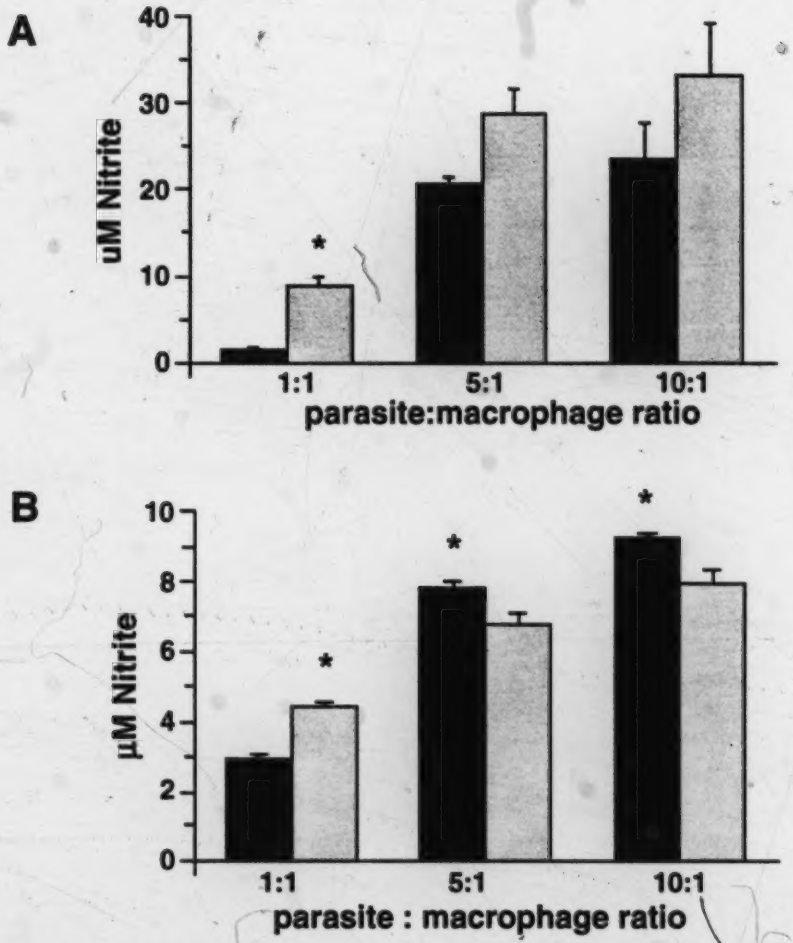

Figure 4.9. Induction of nitric oxide production by goldfish macrophages infected with the protozoan pathogen *L. major* . GMCL (A) or IVDKM (B) were infected with *L. major* promastigotes at the parasite to macrophage ratios listed and cultured in the absence (black) or presence (gray) of MAF (1:4 dilution). Assays were done in polypropylene tubes for GMCL ($5x10^5$ cells/tube) or 1/2 area 96 well culture plates for IVDKM ($5x10^4$ cells/well). Nitrite was determined 96 hrs after cultivation using the Griess reagents. Each data point represents the mean ± SEM of triplicate cultures. (*) $P < 0.05$, comparing presence and absence of MAF in treatment groups.

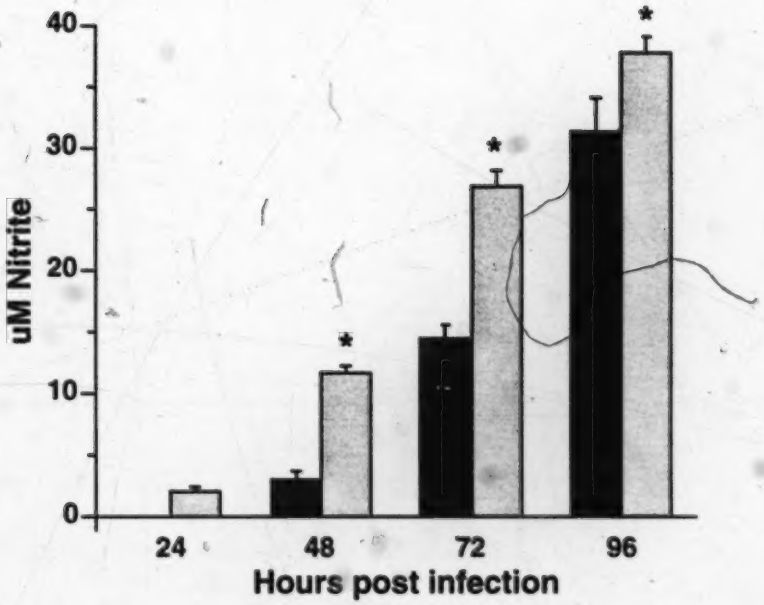

Figure 4.10. Time course of nitric oxide production by GMCL infected with *L. major*. GMCL were seeded into polypropylene tubes ($5x10^5$ cells/tube) and infected with *L. major* promastigotes at a 10:1 parasite to macrophage ratio. Cells were cultured for the specified times in the absence (black) or presence (gray) of MAF. Each data point represents the mean ± SEM of triplicate cultures. (*) $P < 0.05$ comparing the presence and absence of MAF in each treatment group.

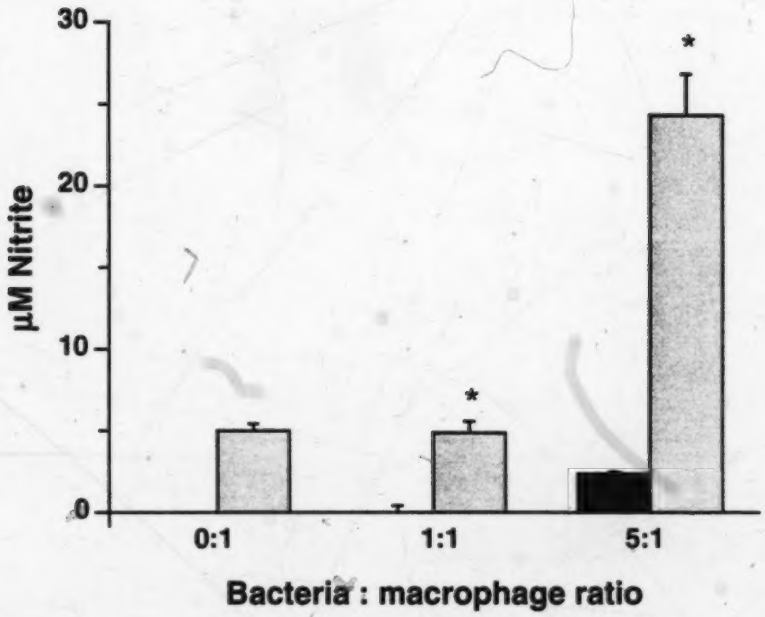

Figure 4.11. Production of nitric oxide by GMCL in response to infection with *M. chelonei* . Macrophages were seeded into polypropylene tubes ($5x10^5$ cells/tube) and infected with *M. chelonei* at the bacteria to macrophage ratios listed, in the presence (grey) or absence (black) of MAF. Nitrite production was determined 72 hrs later. Each data point represents the mean ± SEM of triplicate cultures of a representative experiment out of two that were performed. (*) $P < 0.05$, comparing the presence and absence of MAF in that same treatment group.

CHAPTER V

MECHANISMS REGULATING THE INDUCTION OF THE RESPIRATORY BURST IN GOLDFISH MACROPHAGES [1]

Introduction

Phagocytes, such as macrophages, neutrophils, and eosinophils possess the unique ability to increase oxidative metabolism in response to receptor-mediated phagocytosis of a pathogen: a phenomenon known as the respiratory burst. Induction of the respiratory burst results in the production of numerous toxic oxygen intermediates; including superoxide (O_2-), hydrogen peroxide (H_2O_2), hypochlorous acid ($HOCl^-$), hydroxyl radical (OH·), and peroxynitrite (ONOO-), the antimicrobial actions of which have been well documented (Table 1.1).

Macrophages can be programmed for an enhanced respiratory burst response following stimulation with cytokines or pathogen-derived molecules. These stimulants do not trigger the respiratory burst, but induce the upregulation of enzymatic components necessary for enhanced respiratory burst activity, an effect known as priming. Several cytokines are able to prime the respiratory burst of mammalian macrophages; these include IFN–γ, TNF–α, IL-3, IL-4, M-CSF, and GM-CSF, (Nathan et al., 1983; Wing et al., 1985; Reed et al., 1987; Ding et al., 1988; Cannistra et al., 1988; Phillips and Hamilton, 1989; Phillips et al., 1990; Cassatella et al., 1990; Ho et al., 1992; Laursen et al., 1994; Green et al., 1994). Pathogen-derived molecules such as LPS can also prime the respiratory burst (Pabst and Johnson, 1980; Pabst et al., 1982). Triggering of the respiratory burst

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[1] A version of this chapter has been published: Neumann and Belosevic, 1996. *Dev. Comp. Immunol.*, 20: 427-439.

can be induced by receptor-mediated phagocytosis (Chanock et al., 1994), and by compounds such as phorbol esters (Green and Phillips, 1994).

The respiratory burst has been demonstrated in the phagocytes of lower vertebrates (Chung and Secombes, 1987; Graham and Secombes, 1988; Secombes et al., 1988; Graham and Secombes, 1990 a,b; Secombes and Fletcher, 1992; Hardie et al., 1994; Jang et al., 1995; Tahir and Secombes, 1996), and more recently in several invertebrates (Anderson, 1994; Valembois and Lassegues, 1995; Song and Hseih, 1994), indicating that it is a primitive antimicrobial killing mechanism. However, little information exists regarding the cytokine-induced regulation of respiratory burst responses across diverse taxa. Recent evidence suggests that the cytokine regulation of respiratory burst responses in fish macrophages may be similar to that of mammals (Graham and Secombes, 1988; Graham and Secombes, 1990 a,b). Previous reports have demonstrated that fish macrophages can be primed for an enhanced respiratory burst response following stimulation of macrophages with MAF obtained from mitogen-stimulated kidney leukocytes (Graham and Secombes, 1988; Graham and Secombes, 1990a, 1990b). Priming of teleost macrophage respiratory burst responses require that macrophages be stimulated with MAF for extended cultivation periods (i.e. 48 hrs). The kinetics of priming of fish macrophages appear to be similar to those observed for mammalian macrophages stimulated with supernatants obtained from mitogen-stimulated lymphocytes (Nagakawara et al., 1982; Nathan et al., 1983)

The molecule(s) responsible for priming teleost macrophage respiratory burst responses is a secretory product of T-lymphocytes, which themselves require the presence of macrophages for optimal production (Graham and Secombes, 1990b). The ability of this T-cell-derived molecule to prime the respiratory burst, and its potential to induce nitric oxide production by fish macrophages (Neumann et al., 1995), has led some

authors to speculate that fish may possess an IFN-γ-like molecule (Graham and Secombes, 1990b; Neumann et al., 1995).

This chapter describes results aimed at defining the mechanisms involved in priming goldfish macrophages for enhanced respiratory burst activity. Contrary to the results obtained by others, my results demonstrate that goldfish macrophages undergo a rapid priming of their respiratory burst response when stimulated with MAF. Moreover, this antimicrobial response is selectively deactivated in fish macrophages after reaching maximal potential. This does not represent a general deactivation of macrophages, since macrophages subsequently mount potent nitric oxide responses. My results support a hypothesis that fish macrophages have the capacity to generate sequential antimicrobial responses.

Results

Priming of goldfish macrophage respiratory burst responses with MAF and LPS

To assess the kinetics of priming of the respiratory burst response of fish macrophages, GMCL and IVDKM were stimulated with MAF and/or LPS for various time periods, and triggered for respiratory burst activity using PMA. Respiratory burst potentials were measured using the NBT tetrazolium assay, described in Chapter II.

MAF primed the respiratory burst of GMCL as early as 1 hr after treatment, with maximal priming observed 6 hrs after stimulation (Fig. 5.1). Maximal priming of the respiratory burst response reached a plateau over the next 6 hrs, but declined precipitously by 24 hrs after stimulation, to levels similar to those of unstimulated macrophages. By 48 hrs,

macrophage respiratory burst responses were significantly lower in MAF-stimulated macrophages compared to control macrophages ($P < 0.05$, Fig. 5.1). These results suggest that MAF-activated goldfish macrophages undergo a priming and subsequent "deactivation" of their respiratory burst potentials. MAF, by itself did not induce respiratory burst activity suggesting that deactivation of primed macrophages respiratory burst activity was not a result of a "burned-out" response.

LPS also primed the respiratory burst response of GMCL. However, the kinetics of priming induced by LPS were different than those induced by MAF. LPS induced priming of the respiratory burst as early as 3 hrs after addition to macrophage cultures, and priming of the respiratory burst response continued to increase with increased cultivation periods (Fig. 5.1). Maximal priming of GMCL respiratory burst response with LPS occurred after 48 hrs of treatment (Fig. 5.1).

Co-stimulation of GMCL with MAF and LPS resulted in an enhanced priming of macrophage respiratory burst responses greater than that induced by either factor alone (Fig. 5.1). The enhanced priming effect was dose dependent for both factors (Fig. 5.2). Interestingly, this combined stimulus induced kinetics of priming similar to those observed in GMCL stimulated with MAF only. Maximal priming was observed 6-12 hrs after co-stimulation, but by 24-48 hrs MAF had antagonized the priming effects normally induced by LPS at these same time points (Fig. 5.1). Co-stimulated macrophages had respiratory burst responses similar to the basal respiratory burst responses of unprimed macrophages after 48 hrs of incubation (Fig. 5.1). Thus, although LPS enhanced MAF respiratory burst potentials early after stimulation, its priming potential was antagonized by the deactivation effects induced by MAF after extended cultivation periods.

The kinetics of priming were similar for three different MAF preparations. However, one MAF preparation displayed an impaired

ability to completely antagonize the priming effects of LPS after extensive cultivation periods (compare the kinetics of priming observed in co-stimulated macrophages in Fig. 5.1 and Fig. 5.6.). However, with all MAF preparations tested, co-stimulated macrophages had lower respiratory burst responses at 48 hrs compared to those stimulated for 6 hrs. Moreover, macrophages co-stimulated for 48 hrs had lower respiratory burst potentials compared to macrophages stimulated with LPS only. The results suggest that MAF preparations vary in their ability to completely antagonize the priming effects of LPS, but that they invariably initiate the deactivation of goldfish macrophage respiratory burst responses.

The kinetics of priming induced by MAF and/or LPS were not unique to GMCL, since similar responses were observed using IVDKM cultures (Fig. 5.3). Thus, the rapid priming and subsequent deactivation of goldfish macrophage respiratory burst responses by MAF indicate that this response may represent an important functional mechanism *in vivo*.

The kinetics of priming induced by MAF and/or LPS were also determined for macrophage sub-populations found within GMCL and IVDKM cultures. R2-type macrophages were primed for enhanced respiratory burst responses after only 6 hrs of stimulation with MAF and/or LPS (Fig 5.4). However, unlike IVDKM cultures, the respiratory burst potential of R2-type macrophages continued to increase the longer they were treated with MAF and/or LPS (Fig. 5.4). Thus, MAF-activated R2-type macrophages did not undergo deactivation of their primed respiratory burst response.

These results contrasted those obtained using R3-type macrophages. R3-type macrophages were primed for enhanced respiratory burst responses after only 6 hrs of stimulation with MAF and/or LPS, but subsequently underwent deactivation of their primed state (Fig. 5.4). Thus, R3-type macrophages stimulated with MAF and/or LPS for 48 hrs had significantly lower respiratory burst responses compared to those

stimulated for 6 hrs (Fig. 5.4, $P < 0.05$). This deactivation effect was most notable in R3-type macrophages stimulated with LPS only, where macrophages stimulated for 48 hr had respiratory burst potentials similar to those of unstimulated controls ($P > 0.05$, Fig. 5.4). This deactivation phenomenon did not appear to result from death of the R3 population, since both MAF and LPS induce differentiation of the R3-type macrophage into the more mature R2-type macrophages (Chapter III). Moreover, MAF and/or LPS stimulated R3-type macrophages appeared healthy under phase contrast microscopy.

It is interesting that stimulation of IVDKM (i.e. mixed R2 and R3 sub-populations) with MAF and/or LPS resulted in kinetics of priming that exhibited characteristics of both R2 and R3-type macrophages. Thus, IVDKM cultures behaved like R2-type macrophages when stimulated with LPS only: having increased respiratory burst potentials with increasing stimulation periods. However, IVDKM behaved like R3-type macrophages when MAF was present: becoming rapidly primed for enhanced respiratory burst activity, but subsequently undergoing deactivation of their primed state. The results suggest that these two macrophage sub-populations may affect each other's functional responses in mixed cultures, and that the kinetics of priming observed in IVDKM and GMCL are a result of the cross regulation of the functional activities of R2 and R3-type macrophages that comprise these cultures.

MAF also enhanced the respiratory burst response of LPS-activated GMCL. GMCL pre-treated with LPS for 18 hrs and subsequently stimulated with MAF for 6 hrs had significantly greater respiratory burst responses compared to macrophages stimulated with LPS for 24 hrs (Fig. 5.5, $P < 0.05$). This primed respiratory burst response in co-stimulated macrophages was subsequently deactivated, such that macrophages pre-treated with LPS and stimulated with MAF for 24 hrs had significantly lower respiratory burst responses compared to those stimulated with MAF

for 6 hrs (Fig. 5.5, $P < 0.05$). These results suggest that MAF may act as a culminating signal in priming and deactivation respiratory burst responses of activated goldfish macrophages.

The production of reactive intermediates by activated GMCL was biphasic, with maximal reactive oxygen production occurring 6 hrs after stimulation with MAF and LPS, and maximal nitric oxide production occurring after 24 hrs of stimulation (Fig. 5.6). Therefore, the deactivation of macrophage respiratory burst potentials induced by MAF was not equivalent to an overall deactivation of macrophage antimicrobial activity. This biphasic production of cytotoxic molecules suggests that goldfish macrophages may mount sequential, temporally spaced antimicrobial effector mechanisms.

Priming of goldfish macrophage respiratory burst responses by autocrine mediators

Recent evidence also demonstrated that the respiratory burst of teleost macrophages can be primed by autocrine mediators. To determine whether autocrine mediators could enhance respiratory burst activity of goldfish macrophages, GMCL were pulsed with MAF, and supernatants produced by these cytokine-activated macrophages used to prime the respiratory burst of separate GMCL cultures. GMCL were pre-treated with these supernatants for 0, 6, or 12 h before the addition of MAF, in order to determine whether factors produced by activated macrophages were responsible for the deactivation effects observed. Respiratory burst responses were measured 6 h after the addition of MAF.

MAF-activated macrophages produced autocrine factors which primed GMCL for enhanced respiratory burst activity, regardless of their pre-treatment condition (Fig. 5.7). Moreover, GMCL stimulated with

macrophage-derived autocrine factors, by themselves, also had enhanced respiratory burst potentials (Fig. 5.8).

Discussion

This chapter describes the results of a novel phenomenon of macrophage activation: a sequential induction of temporally spaced antimicrobial killing mechanisms by teleost macrophages. Goldfish macrophages displayed a biphasic production of cytotoxic molecules, with maximal reactive oxygen production occurring after only 6-12 hrs of stimulation with MAF and/or LPS, and maximal reactive nitrogen production occurring after 24 hrs of stimulation with these activators. Although the respiratory burst of goldfish macrophages could be rapidly primed with MAF (i.e. 6 hrs), these cells subsequently underwent deactivation of their enhanced respiratory burst state. This deactivation event did not represent a classical deactivation of macrophage antimicrobial responses, since it did not affect the ability of activated macrophages to produce nitric oxide. A recent report by Mulero and Meseguer (1998) also demonstrated that MAF and LPS co-stimulated gilthead seabream macrophages have reduced respiratory burst responses at 48 hrs compared to macrophages stimulated with MAF only. Unfortunately, all respiratory burst assays were carried out after 48 hrs of cultivation in their study, making it impossible to determine whether macrophages first underwent rapid priming of their respiratory burst followed by subsequent deactivation.

The sequential induction of antimicrobial responses observed in GMCL and IVDKM cultures results from the distinct effects that MAF and/or LPS have on the activation kinetics of the specific sub-populations

of macrophages that comprise these cultures. R2-type macrophages increased respiratory burst potentials in response to longer stimulation periods with these stimulants. R3-type macrophages rapidly increased their respiratory burst potential in response to stimulation with MAF and/or LPS (i.e. 6 hrs), but subsequently underwent deactivation of their primed state. Neither R2 or R3-type macrophages completely mimicked the kinetics of priming observed in activated IVDKM or GMCL cultures. LPS activated IVDKM and GMCL increased the respiratory burst response with increasing cultivation periods, a characteristic shared by R2-type macrophages. However, the respiratory burst response of IVDKM and GMCL were rapidly primed in response to stimulation with MAF, and subsequently deactivated, a characteristic shared by R3-type macrophages. The results indicate that R2 and R3-type macrophage sub-populations may actively cross-regulate each other's antimicrobial activities *in vitro*. The ability of a specific macrophage sub-population to regulate the respiratory burst of another macrophage sub-population has not been previously documented. This cross-regulation of the antimicrobial responses may be conclusively determined through experiments using mixed cultures of sorted macrophage sub-populations (i.e. R2 and R3-type macrophages). Sorted sub-populations of macrophages can be combined in definite proportions to see what effect a "contaminating" sub-population may have on the "dominant" macrophage sub-population. It is possible that this cross-regulation of antimicrobial functions between macrophage sub-populations results in the induction of temporally spaced antimicrobial mechanisms of fish macrophages.

The R3-type macrophage (putative monocyte) appears to play an important role in the regulation of the sequential induction of goldfish macrophage antimicrobial effector mechanisms. As such, goldfish monocytes *in vivo* may regulate the duration of expression of reactive oxygen intermediates produced by activated tissue macrophages (and

potentially granulocytes) at the site of inflammation. Although monocytes may be recruited from the circulation to augment the initial production of reactive intermediates (i.e. because they are rapidly primed for enhanced respiratory burst activity), their accessory role may be to selectively deactivate this mechanism at inflammatory loci. Their selective deactivation of respiratory burst response would minimize unnecessary tissue destruction but permit the subsequent production of nitric oxide by more mature tissue macrophages.

The induction of sequential macrophage antimicrobial responses may be of utmost significance in host defense against pathogens. Pathogenic organisms that are susceptible to respiratory burst products are rapidly killed upon phagocytosis by macrophages. Pathogens that survive this initial oxidative assault (as a result of possessing detoxifying enzymes such as superoxide dismutase or catalase), may be susceptible to subsequent antimicrobial attacks mounted by activated macrophages (i.e. nitric oxide, peroxynitrite, tryptophan degradation, iron deprivation). Deactivation of primed respiratory burst responses would minimize tissue damage in an otherwise futile attempt to eliminate reactive oxygen insensitive pathogens. Moreover, the coordination of temporally arranged antimicrobial responses would maximize the effectiveness of each individual response, allowing macrophages to divert their metabolic energy into a single, extremely potent, but short-lived antimicrobial assault.

Due to the crude MAF preparation used in my experiments it is impossible to attribute the pattern of priming observed to a single cytokine, and as such, other factors found in the crude MAF preparations may play a role in regulating the various kinetics of priming observed in goldfish macrophages. However, crude preparations of mammalian MAF, prepared from mitogen-stimulated lymphocytes, display respiratory burst priming kinetics similar to that induced by recombinant IFN-γ, suggesting

that IFN-γ is the main mediator of mammalian lymphokine MAF activity (Nagakawara et al., 1982; Nathan et al., 1983). Data presented in Chapter VI suggest that a single cytokine may be responsible for the rapid priming and subsequent deactivation of goldfish macrophage respiratory burst response. Nevertheless, it is the complex network of cytokine interactions that results in a finely tuned but highly effective immune response. Characterization of the molecules present in crude MAF preparations that selectively activate macrophage antimicrobial responses is described in Chapter VI.

It is interesting that the R2-type goldfish macrophage displays the kinetics of MAF-induced priming that have been reported by others. Rainbow trout and dab (*Limanda limanda*) resident kidney macrophages require extensive incubations with MAF (i.e. 48 hrs) for priming their respiratory burst response (Chung and Secombes, 1987; Graham and Secombes, 1988; Graham and Secombes, 1990 a,b; Hardie et al., 1994; Francis and Ellis, 1994; Marsden et al., 1994; Jang et al., 1995; Tahir and Secombes, 1996). Unique to this study is the observation that R3-type macrophages can undergo rapid priming and deactivation of respiratory burst potentials, and that they may modulate the activities of more mature macrophage sub-populations.

The results described in this chapter further support the recent finding that macrophage-derived autocrine factors can enhance teleost macrophage respiratory burst responses (Jang et al., 1995). In addition, the kinetics of priming induced by LPS, observed in my study, are similar to those reported by others, where maximal priming occurs after extensive cultivation periods (48 hrs to several days, Solem et al., 1995)

The multi-component enzyme responsible for respiratory burst activity, the NADPH oxidase or the respiratory burst oxidase, is similar between the different phagocytic cell types (Chanock et al., 1994), but regulation of its activity (i.e. affinity for substrate), or initiation of

transcription of its multiple gene products is differentially regulated (Cassatella et al., 1990). Whereas maximal priming effects are observed after 48 hrs of stimulation of murine macrophages with IFN–γ, neutrophils are primed for maximal respiratory burst activity within hours of exposure to this cytokine (Newberger et al., 1988; Cassatella et al., 1990). Likewise, mRNA transcription products of the gp91phox subunit of the respiratory burst oxidase can be detected much earlier in neutrophils than in macrophages after exposure to IFN–γ (Cassatella et al., 1990). However, there is evidence to suggest that mammalian macrophages, under specific circumstances, can be induced to undergo rapid priming of their respiratory burst response. Murine macrophages can undergo rapid priming of their respiratory burst in response to stimulation with LPS or other pathogen-derived molecules (Lin et al., 1993). Interestingly, human monocyte respiratory burst potentials can be rapidly primed (i.e. 30 min) in response to stimulation with IFN-γ and/or TNF-α (Kumaratilake et al., 1990). Taken together, these observations suggest that the regulation of respiratory burst responses in vertebrate macrophages by cytokines and pathogen-derived molecules may be very similar.

The results described in this chapter raise interesting questions regarding the regulation of cytokine-induced macrophage cytotoxic mechanisms, and provide insight for examining the sequential regulation of macrophage antimicrobial mechanisms in all classes of vertebrates.

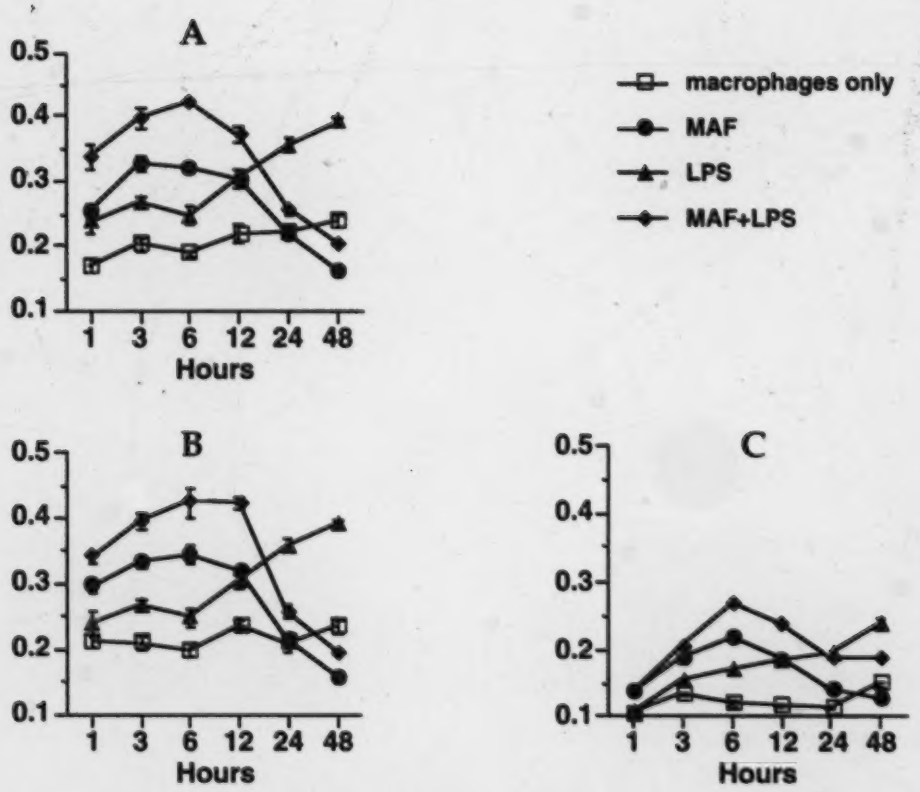

Figure 5.1. Kinetics of MAF and/or LPS-induced priming of the respiratory burst response of GMCL. Macrophages ($2x10^5$ cells/well) were seeded into wells of a 96 well culture plate and stimulated with MAF (1:3 dilution) and/or LPS (5 μg/ml) for the specified times. GMCL were subsequently triggered for respiratory burst activity using PMA, and respiratory burst potentials measured using the NBT assay. Three different MAF samples were used in each of the experiments shown above (A, B, and C). Each point represents the mean optical density ± SEM of six cultures in A and B, and four cultures in C.

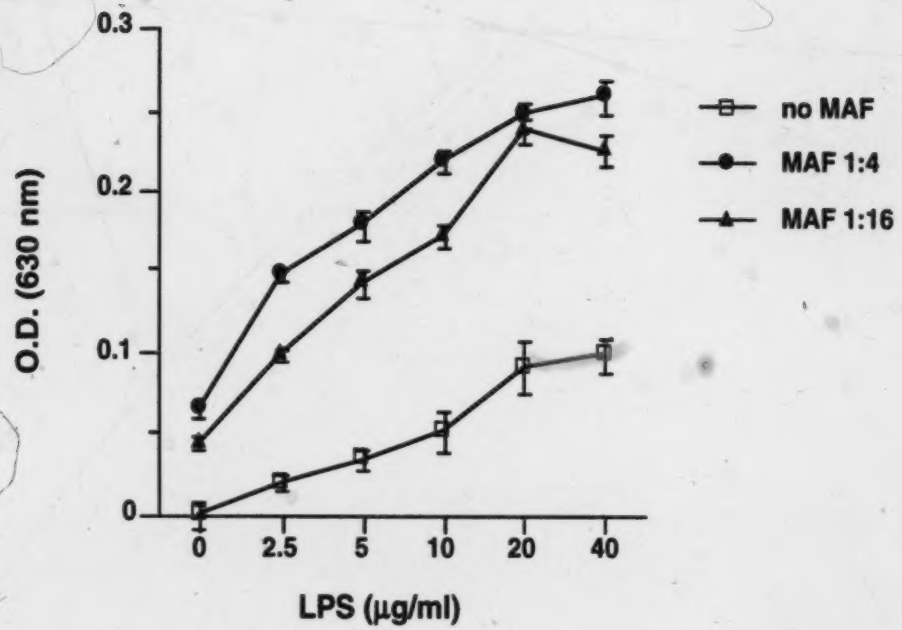

Figure 5.2. MAF and/or LPS-induced priming of the respiratory burst response of GMCL. Macrophages were seeded into wells of a 96 well culture plate ($2x10^5$ cells/well) and stimulated with various concentrations of MAF and/or LPS. PMA-triggered respiratory burst responses were measured 6 hrs later. The data are from a representative experiment of two that were performed. Each point represents the mean optical density of quadruplicate cultures ± SEM.

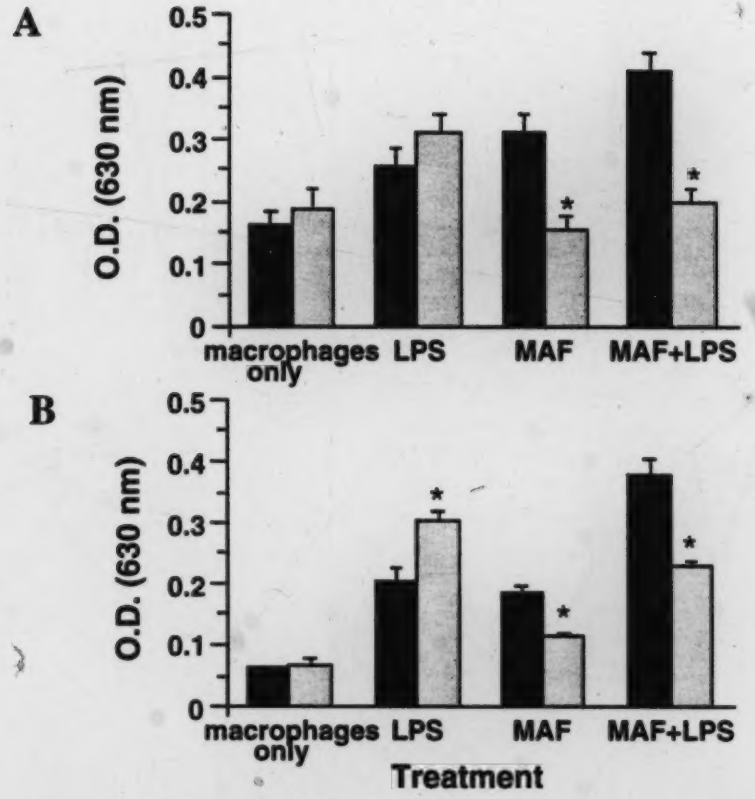

Figure 5.3. Priming the respiratory burst of IVDKM by MAF and/or LPS. IVDKM were established using GMCL-derived CCM (A) or KL-derived CCM (B). IVDKM were stimulated with MAF (1:3 dilution) and/or LPS (5 µg/ml for A, and 1 µg/ml for B) for 6 hrs (black) or 48 hrs (gray). PMA-triggered respiratory burst responses were measured at these time points using the NBT assay. In graph "A", data represents the mean ± SEM of macrophage cultures established from at least eight fish. In graph "B", IVDKM from 4 fish were pooled and bars represents the mean ± SEM of five replicate cultures.

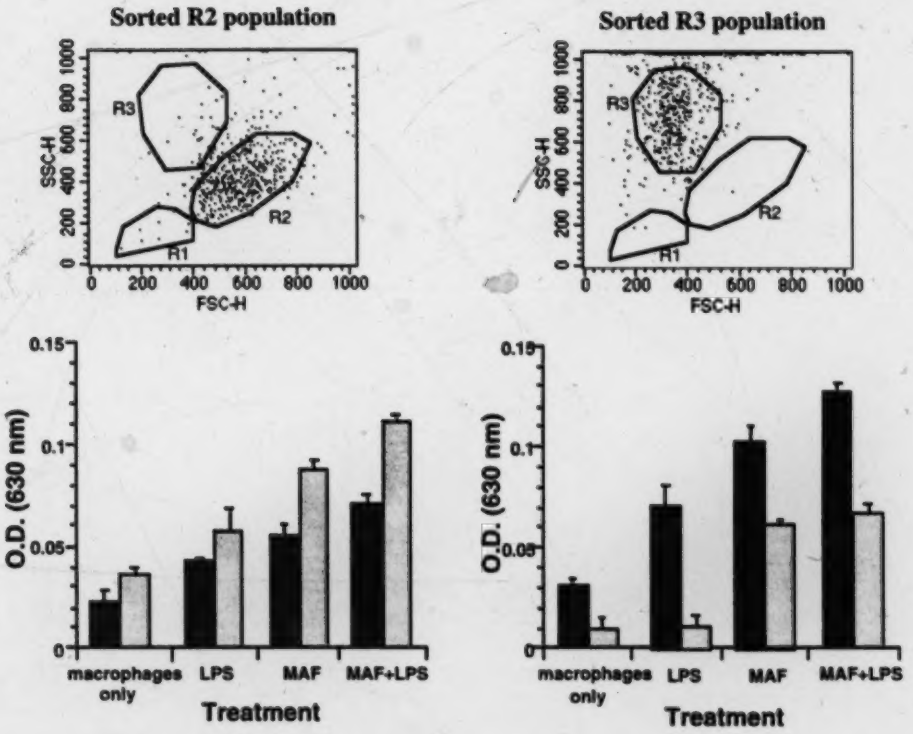

Figure 5.4. Priming of the respiratory burst response of R2 and R3-type macrophages with MAF and/or LPS. Macrophage sub-populations were sorted from IVDKM cultures using flow cytometry and seeded into wells of half area 96 well culture plates ($2.5x10^4$ cells/well). Macrophages were stimulated with MAF (1:3 dilution) and/or LPS (1 μg/ml) for 6 hrs (black) or 48 hrs (gray). PMA-triggered respiratory burst responses were determined using the NBT assay. Each bar represents the mean ± SEM of triplicate cultures. The data are representative of two experiments that were performed for each macrophage sub-population. (*) $P<0.05$, comparing O.D. values obtained from 6 hr and 48 hr incubations of the same treatment group.

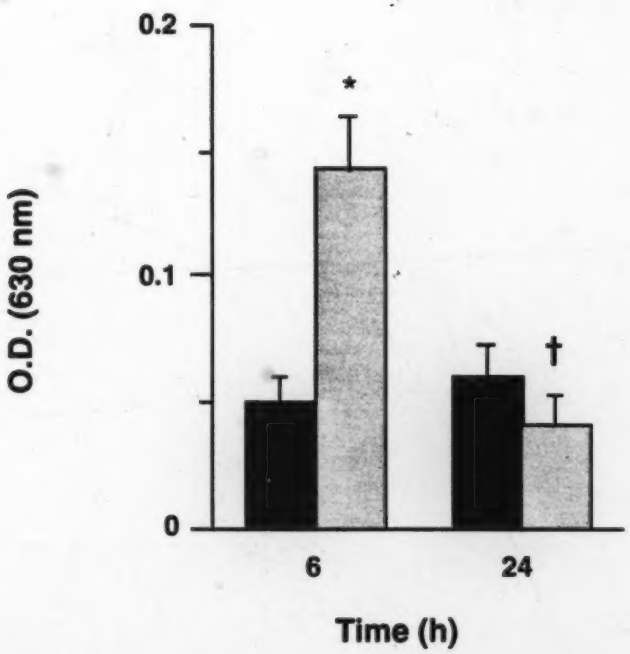

Figure 5.5. Ability of MAF to prime and subsequently de-prime the respiratory burst response of LPS-activated GMCL. Macrophages were pretreated with 5μg/ml LPS for 18 hrs, and then treated with either medium only (black) or a 1:4 dilution of MAF (gray). PMA-triggered respiratory burst responses were measured 6 or 24 hrs after the addition of MAF, using the NBT tetrazolium assay. Each bar represents the mean ± SEM of quadruplicate cultures, blanked against basal respiratory burst levels of unstimulated macrophages at each time point. The data are from a representative experiment out of two that were performed. (*) $P < 0.05$, comparing O.D. values of macrophages cultured in the presence or absence of MAF at that same time point. (†) $P < 0.05$, comparing O.D. values of macrophage stimulated with MAF for 6 or 24 hrs.

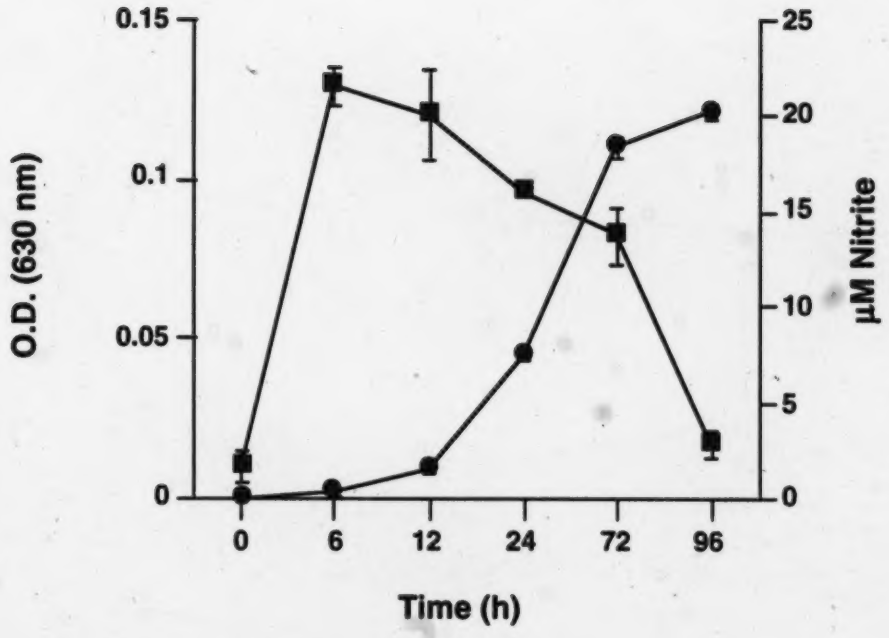

Figure 5.6. Biphasic production of reactive oxygen and nitrogen intermediates by GMCL. Macrophages were treated with MAF (1:4 final dilution) and LPS (5 µg/ml), and production of superoxide (squares) and nitrite (circles) monitored for 96 hrs. Each data point represents the mean ± SEM of quadruplicate cultures, blanked against the basal levels of reactive intermediates produced by unstimulated macrophages. The data are representative of two separate experiments that were performed.

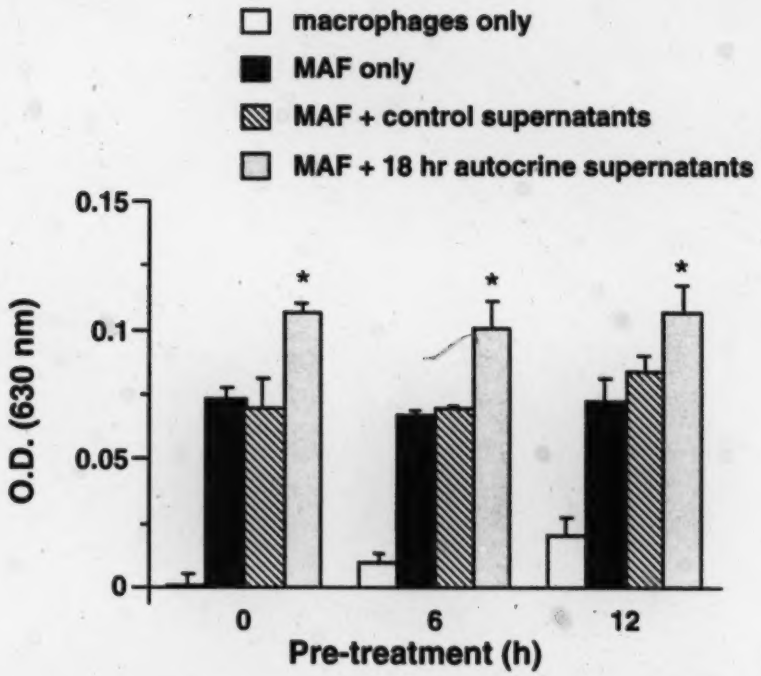

Figure 5.7. Priming of the respiratory burst response of GMCL by autocrine factor(s) produced by activated GMCL. In separate cultures, GMCL were stimulated with MAF for 6 h, washed to remove the MAF stimulus, and supernatants collected from these MAF-pulsed macrophages after an additional 18 h of cultivation. Control supernatants were removed immediately after washing to ensure complete removal of MAF. In separate wells, GMCL ($2x10^5$ cells/well) were pre-treated with these autocrine supernatants (1:3 dilution) for the time periods specified. Medium only was added to the untreated macrophage group. After pre-treatment, GMCL were stimulated with medium (untreated macrophages) or MAF (1:4 dilution in all other groups). PMA-triggered respiratory burst responses were measured 6 h after the addition of MAF, using the NBT assay. Each bar represents the mean ± SEM of quadruplicate cultures and are representative of two experiments that were performed. (*) $P < 0.05$ comparing macrophages treated with MAF and control or autocrine supernatants.

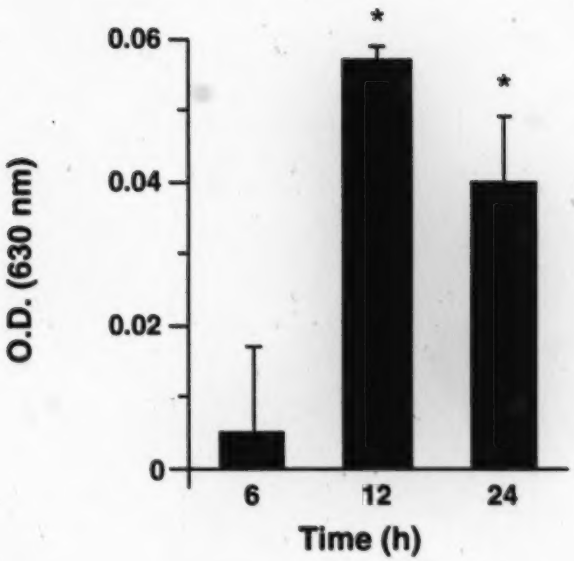

Figure 5.8. Priming of the respiratory burst of GMCL by autocrine factor(s). Macrophages were stimulated with autocrine supernatants for the time specified, and then assayed for PMA-triggered respiratory burst activity. The bars represent the mean ± SEM of quadruplicate cultures, blanked against levels of unstimulated macrophages at the same time points. The data are representative of two experiments that were performed. (*) $P < 0.05$, comparing respiratory burst response between treated macrophages and controls.

CHAPTER VI

BIOCHEMICAL AND FUNCTIONAL CHARACTERIZATION OF MAF SECRETED BY MITOGEN-STIMULATED GOLDFISH KIDNEY LEUKOCYTES

Introduction

There are no purified or recombinant cytokines available from fish. Consequently, the characterization of teleost MAF has been restricted to functional studies that have examined induction of macrophage antimicrobial responses by crude cytokine preparations. These crude cytokine preparations are obtained from the culture supernatants of mitogen-stimulated kidney leukocytes. Although these crude cytokine preparations induce macrophage antimicrobial responses, the plethora of soluble mediators produced by stimulated leukocytes makes it impossible to attribute macrophage activation, or the kinetics of activation, to a single molecule.

It is well established that mitogen-stimulated mammalian lymphocytes secrete a MAF that induces kinetics of macrophage activation similar to recombinant IFN-γ (Nagakawara et al., 1982; Nathan et al., 1983), suggesting that IFN-γ is the primary mediator of MAF activity in mitogen-stimulated mammalian lymphocyte supernatants. The kinetics of macrophage activation observed for mammalian lymphocyte-derived MAF are similar to those reported using kidney leukocyte-derived MAF of teleosts. In both species, MAF produced through mitogenic stimulation of leukocytes primes the respiratory burst response of macrophages after 48 hrs of stimulation (Nagakawara et al., 1982, Graham and Secombes, 1988, 1990 a,b). In the case of teleosts, the MAF produced is T-cell-derived, heat and acid labile, and induces respiratory burst activity (Graham and

Secombes, 1990a); characteristics that are all shared by mammalian IFN-γ. MAF produced through the mitogenic stimulation of kidney leukocytes also induces nitric oxide production in teleost macrophages (Neumann et al., 1995; Yin et al., 1997; Mulero and Meseguer, 1998). The available information suggests that fish may possess the molecular equivalent of mammalian IFN-γ, and that this molecule may be the primary mediator of MAF activity in crude cytokine preparations.

However, teleost IFN-γ may not be the only MAF present in crude cytokine preparations. Human TNF-α (hTNF-α) has been shown to augment the respiratory burst response of rainbow trout macrophages, and monoclonal antibodies to the high affinity hTNF-α receptor impaired the ability of kidney leukocyte-derived MAF supernatants to prime teleost macrophage respiratory burst responses (Hardie et al., 1994; Jang et al., 1995b; Tahir and Secombes, 1996; Campos-Perez et al., 1997). Moreover, the addition of recombinant high affinity receptor for hTNF-α to crude MAF preparations impaired priming of the respiratory burst response of rainbow trout macrophages (Jang et al., 1995b). These data suggest that fish may also possess the molecular equivalent of mammalian TNF-α, and that this molecule is also a MAF component of crude cytokine preparations.

Although teleost IFN-γ and TNF-α may be the primary mediators of MAF activity in crude cytokine preparations, other MAFs present in these preparations may modulate the macrophage activating properties of these two cytokines. Cytokine activities resembling interferon-α (IFN-α), IL-1 and IL-2 have also been reported from fish. These cytokines are known to be important modulators of macrophage activation in mammals (see Chapter I). However, the roles that teleost IFN-α, IL-1 and IL-2 play in modulating the induction of macrophage antimicrobial mechanisms has yet to be determined.

It has been demonstrated by several groups that fish cells secrete an IFN-like molecule in response to viral infection (De Kinkelin and Dorson, 1973; De Sena and Rio, 1975; Eaton, 1990; Dorson et al., 1992; Congleton and Sun, 1996). Cloning and expression of a flatfish (*Paralichthys olivaceus*) interferon-α-like molecule has been recently reported (Tamai et al., 1993), although the lack of amino acid sequence homology to mammalian IFNs (approximately 10%) and lack of species specificity has lead others to speculate that it may not be an IFN (Secombes et al., 1996).

Teleost leukocytes have also been shown to secrete an IL-1-like factor. Carp (*Cyprinus carpio*) macrophages and granulocytes secrete a factor that supports proliferation of a murine T-cell line (D10.G4.1), a cell line dependent upon exogenously added IL-1 for sustained growth (Elsaesser and Clem, 1994; Verberg-van Kemenade et al., 1995; Weyts et al., 1997). Proliferation induced by this leukocyte-derived factor is inhibited by polyclonal sera raised against murine IL-1 (Elsaesser and Clem, 1994; Verberg-van Kemenade et al., 1995). Other reports have also demonstrated bioactivity of human IL-1 on fish lymphocytes (Siegel et al., 1986; Hamby et al., 1986). Murine IL-1 administered *in vivo* to tilapia induces an acute phase-like reaction and fever (Balm et al., 1995). It is interesting that IL-1-like activities have also been reported from invertebrates, such as tunicates (Beck et al., 1989) and echinoderms (Beck and Habicht, 1986), suggesting that this cytokine is highly conserved.

Carp kidney leukocytes stimulated with phytohemagglutinin secrete an IL-2-like factor that induces proliferation of carp thymocytes (Grondel and Harmsen, 1984; Caspi and Avtalion, 1984; Watkins and Cohen, 1985). An IL-2 like molecule has been recently cloned and sequenced from flatfish (Tamai et al., 1993).

The only other molecule known to modulate teleost macrophage antimicrobial responses is growth hormone (GH). Recombinant rainbow trout growth hormone has been shown to prime sea bass macrophage

respiratory burst response after 24 hrs of stimulation (Munoz et al., 1998; Calduch-Giner, et al., 1997). Furthermore, administration of recombinant chum salmon GH to rainbow trout *in vivo* enhanced the survival of fish infected with *Vibrio anguillarum* (Sakai et al., 1997).

It is clear that induction of macrophage activation in mammals is regulated by a complex network of interacting cytokines. Similarly, the induction of teleost macrophage activation by crude MAF preparations may be regulated through the concerted actions of multiple cytokines present in these preparations. The purpose of this chapter was to characterize the mediators present in crude MAF preparations that have the ability to modulate the antimicrobial responses of goldfish macrophages.

Results

Analysis of crude MAF preparations by gel permeation FPLC (GP-FPLC)

Crude MAF preparations were fractionated by molecular size using GP-FPLC, to determine whether MAFs of distinct size were present in crude cytokine preparations. Individual fractions collected from GP-FPLC were tested for their capacity to induce nitric oxide production or prime respiratory burst responses of GMCL or IVDKM.

Induction of nitric oxide production

Fractionation of crude MAF preparations by GP-FPLC resulted in the isolation of a MAF (approximate MW= 50 kD), that induced nitric oxide production in both GMCL and IVDKM (Figs. 6.1 and 6.2). Attempts were made to estimate the quantitative recovery of this molecule after GP-

FPLC; however, the GP-FPLC partially purified 50 kD MAF occasionally induced greater production of nitric oxide than did the original crude MAF samples (and vice versa). In some instances, goldfish macrophages stimulated with crude MAF samples produced little or no nitric oxide, whereas cells in parallel cultures stimulated with the GP-FPLC partially purified 50 kD MAF produced large quantities of nitric oxide, irrespective of the dose (Fig. 6.3). The inability to obtain stable quantitative measurements of nitric oxide using crude MAF preparations made it impossible to accurately estimate the recovery of MAF activity after GP-FPLC.

The variability in nitric oxide inducing activity of crude MAF preparations may be explained by two possible postulates: 1) the relative proportions of R2 and R3-type macrophages within individual IVDKM cultures (Table 3.1) may influence the overall production of nitric oxide in a given culture (i.e. cultures predominated by R3-type macrophages would produce low nitric oxide); or 2) macrophage deactivators present in crude cytokine preparations may influence nitric oxide output by activated macrophages. Thus, fractionation of crude cytokine preparations may segregate MAF activity away from macrophage deactivator activity, resulting in more potent nitric oxide responses in fractionated samples.

To test the latter hypothesis, IVDKM were pre-treated for 6 hrs with fractions collected from GP-FPLC fractionated crude cytokine preparations. IVDKM were subsequently co-stimulated with the GP-FPLC partially purified 50 kD MAF and LPS, in order to induce nitric oxide production in IVDKM. The rationale behind this experiment was to allow any macrophage deactivating factor, fractionated from crude cytokine preparations using GP-FPLC, to be presented to the macrophage before the activating signal (i.e. the 50 kD MAF and LPS). The desired effect would manifest itself as an antagonized nitric oxide response (i.e. decreased nitric oxide output). The results of this experiment are shown in Figure 6.4. A

distinct nitric oxide deactivating activity was observed in fraction 39, and corresponded to a molecule of approximately 12 kD in MW (Fig. 6.4). Deactivation of the macrophage nitric oxide response does not appear to be a result of a decrease in viability of macrophages since macrophages appear healthy under phase contrast microscopy. Moreover, if the deactivator (fraction 39) is presented to the macrophage after the activating signal then nitric oxide production is enhanced (Stafford, Neumann and Belosevic, unpublished data). Together, the results suggest that the kinetics and production of nitric oxide by goldfish macrophages *in vitro* is regulated by the balance of both macrophage activating and deactivating factors present in crude MAF samples.

Since macrophage activators and deactivators were present in crude MAF preparations, it seemed plausible that R2 and R3-type macrophage antimicrobial function may be differentially regulated by these two activities (Figure 4.8). Thus, R2-type macrophages may produce nitric oxide because they are responsive to the 50 kD MAF, and/or are relatively unresponsive to deactivation by the 12 kD molecule. Conversely, R3-type macrophages may not produce nitric oxide when stimulated with crude cytokine preparations because of the presence of the 12 kD deactivator. However, R3-type macrophages produced little or no nitric oxide when cultured in the presence of the GP-FPLC partially purified 50 kD MAF (Table 6.1). These data support my original hypothesis, that nitric oxide production may be an acquired characteristic of more mature macrophage sub-populations (see Chapter IV).

Priming of the respiratory burst response

The partially purified 50 kD MAF that induced nitric oxide production in goldfish macrophages also rapidly primed (i.e. 6 hrs) the respiratory burst response of goldfish macrophages (Fig. 6.5). However, unlike the sharp peak activity obtained using the nitric oxide bioassay,

multiple peak activity was observed from GP-FPLC fractions using the respiratory burst bioassay as a measure of macrophage activation (compare Figs. 6.2 and 6.5). Interestingly, distinct MAF activities could be identified within this broad peak if the duration of stimulation was modified. Thus, macrophage cultures were stimulated with GP-FPLC purified fractions for 6 or 24 hrs and respiratory burst activity measured at these time points.

The GP-FPLC partially purified 50 kD MAF induced priming of goldfish macrophage respiratory burst responses after only 6 hrs of stimulation; however, this primed respiratory burst response was subsequently de-primed after 24 hrs of stimulation (Fig. 6.6a). The kinetics of priming induced by this molecule were similar to that observed after stimulation of goldfish macrophages with crude MAF preparations (see Fig. 5.1).

A 30 kD molecule, partially purified using GP-FPLC, also primed goldfish macrophage respiratory burst responses after only 6 hrs of stimulation (Fig. 6.6). However, this molecule continued to increase macrophage respiratory burst responses after 24 hrs of stimulation. Interestingly, the kinetics of priming induced by this molecule were similar to that observed after stimulation of goldfish macrophages with LPS only (see Fig. 5.1). Moreover, the GP-FPLC partially purified 30 kD MAF induced significantly less production of nitric oxide in goldfish macrophages than did the 50 kD MAF ($P < 0.05$, Fig. 6.6b).

Purification of the 50 kD MAF by chromatofocusing FPLC (C-FPLC)

The 50 kD MAF partially purified using GP-FPLC was subsequently subjected to C-FPLC in an attempt to purify this MAF to homogeneity. The 50 kD molecule was specifically chosen for further purification

because of its ability to induce nitric oxide production, and to prime/de-prime macrophage respiratory burst responses.

C-FPLC was initially done in shallow 7.5 - 5.0 pH gradients. A distinct nitric oxide inducing activity fractionated to the pre-gradient volume, indicating that the 50 kD MAF was a relatively basic molecule (pI > 7.5, Fig. 6.7). This molecule by itself induced little or no priming of the respiratory burst in goldfish macrophages, but could significantly enhance the rapid priming of macrophage respiratory burst responses induced by the partially purified 30 kD MAF (Fig. 6.8). Moreover, the 50 kD MAF initiated the de-priming of primed respiratory burst responses in macrophages co-stimulated with both factors after extended cultivation periods (i.e. 48 hrs, Fig. 6.8).

The GP-FPLC partially purified 50 kD MAF was subsequently chromatofocused using more basic pH gradients (pH 9.3 - 6.0), in an attempt to resolve the pI of this molecule. Interestingly, two nitric oxide inducing activities were observed in the pre-gradient volumes when C-FPLC was done using pH gradients of 9.3 - 6.0 (Fig. 6.9), indicating that the molecules responsible for induction of nitric oxide were very basic (pI > 9.3). The delayed elution of the second MAF indicates that this molecule has a pI closer to 9.3 than the first MAF. It is this characteristic that allows the more basic gradient to resolve two nitric oxide inducing activities compared to only a single activity when pH gradients of 7.5 - 5.0 were used.

SDS-PAGE of GP-FPLC and C-FPLC purified MAFs

Molecules fractionated from crude cytokine preparations using GP-FPLC and C-FPLC were subsequently analyzed by SDS-PAGE in order to determine the relative purity of fractionated MAF samples. Particular emphasis was placed on the 50 kD MAF for analysis using SDS-PAGE. GP-

FPLC fractions containing the most potent nitric oxide inducing activity (fractions 30-39, with maximal activity in fractions 33 and 34) were separated on SDS-PAGE to determine whether the presence of a specific protein correlated with increased MAF activity. Under non-reducing conditions two doublets of 59 kD and 55 kD, and 35 kD and 33 kD, were observed that fit these criteria (Fig. 6.10).

SDS-PAGE was performed on the two 50 kD MAFs purified by GP-FPLC and C-FPLC (pH gradient 9.3 - 6.0). Under non-reducing conditions two doublets of 59 kD/ 55 kD, and 35 kD/ 33 kD, were observed in the first MAF to elute from C-FPLC (Fig. 6.11, lane 2). Interestingly, the second MAF also contained the same doublet of 59 kD/ 55 kD, but not the smaller doublet. However, a single band of 33 kD was also observed in the second MAF (Fig 6.11, lane 1). Under reducing conditions, a single doublet of 72 kD/ 67 kD was observed for both MAFs (Fig. 6.11).

Discussion

The results presented in this chapter demonstrate that crude cytokine preparations contain a plethora of molecules that can modulate teleost macrophage antimicrobial responses. Both macrophage activating and deactivating activities were fractionated from crude cytokine preparations using gel permeation and chromatofocusing FPLC.

Multiple MAF activities were identified by FPLC based on the kinetics of activation and the types of macrophage antimicrobial responses that they induced. Two MAF activities, corresponding to an approximate molecular weight of 30 kD and 50 kD, were identified. A 30 kD MAF, partially purified using GP-FPLC, primed macrophage respiratory burst responses, but only induced minor production of nitric oxide. The priming potential of the 30 kD MAF correlated with the duration of

stimulation (i.e. longer stimulation periods induced greater priming of the respiratory burst). The kinetics of priming induced by the 30 kD molecule were similar to those observed when goldfish macrophages were stimulated with LPS only. This would suggest that the 30 kD MAF may be a product of LPS-stimulated macrophages, and potentially equivalent to TNF-α. TNF-α is a potent MAF produced by LPS-stimulated macrophages, and is able to prime mammalian macrophages for enhanced respiratory burst responses (Chapter I). Human recombinant TNF-α has been shown to prime rainbow trout macrophage respiratory burst responses, and the addition of soluble human recombinant TNF-α receptors to crude MAF preparations impairs the ability of these crude preparations to prime macrophage respiratory burst responses (Jang et al., 1995; Tahir and Secombes, 1996; Campos-Perez et al., 1996). However, since crude cytokine preparations are obtained from kidney leukocyte cultures (i.e. cultures containing multiple cell types), it is premature to attribute a cellular source or molecular homologue to this partially purified molecule.

However, my observation that a 30 kD MAF primes teleost macrophage respiratory burst activity supports the findings of others. Graham and Secombes (1990b) isolated MAF activities from crude MAF preparations using HPLC, the actions of which corresponded to proteins having molecular weights of 19 kD and 32 kD. Moreover, the kinetics of activation induced by the 30 kD MAF, described in my study, are similar to what has been observed by this group (Graham and Secombes, 1990 a,b), in that the priming potential of the 30 kD MAF increases with longer stimulation periods.

Unlike the 30 kD MAF, the novel 50 kD MAF induced potent nitric oxide production in goldfish macrophages, but induced little or no priming of the respiratory burst. However, the 50 kD MAF could significantly enhance priming of the respiratory burst induced by the 30 kD MAF during short co-stimulation periods (6-18 hrs). Longer co-

stimulation periods initiated de-priming of the primed macrophage respiratory burst responses. The 50 kD MAF augmented and accelerated the priming effects of the 30 kD MAF, but antagonized the priming effects induced by the 30 kD MAF after extended stimulation periods. Thus, the 50 kD MAF appears to be responsible for the kinetics of activation observed in goldfish macrophages stimulated with crude MAF preparations (see Chapter V). A summary of the known macrophage modulating activities present in crude cytokine preparations is given in Table 6.2.

The 50 kD MAF partially purified by GP-FPLC was fractionated into two distinct nitric oxide-inducing activities using basic pH gradients in C-FPLC (pH 9.3 - 6.0). SDS-PAGE revealed that these two activities contained almost identical proteins. Under non-reducing conditions the more basic 50 kD MAF was comprised of two doublets, a 59 kD/ 55 kD doublet, and a 35 kD/ 33 kD doublet. The less basic 50 kD MAF was comprised of a single doublet (59 kD/ 55 kD) and a single band corresponding to 33 kD. Thus, these two MAFs differed only by the absence of the 35 kD band in the less basic form. However, under reducing conditions, a single doublet of 72 kD/ 67 kD was observed for both of these MAFs, indicating that the two 50 kD MAFs are similar. It is interesting that the 35 kD/33 kD doublet is approximately half the molecular weight of the 59 kD/55 kD doublet. It is possible that the active molecule may either represent a cleaved product of the 59 kD/ 55 kD doublet (i.e. the active MAF is the 35 kD/33 kD doublet), or that the active molecule is a homodimer/ heterodimer of the 35 kD/33 kD doublet (i.e. the active MAF is the 59/55 kD doublet).

My results suggest several fundamentally important roles that the 50 kD MAF may play in host defense against pathogens. First, and foremost, this MAF rapidly activates macrophages for enhanced killing, as demonstrated by its ability to accelerate cytokine induced priming of the respiratory burst response of goldfish macrophages (i.e. priming induced

by the 30 kD MAF). The ability of macrophages to respond quickly to infection is crucial for preventing disseminated disease states. It is particularly important for macrophages to hastily kill pathogens that utilize them as a host cell for maintenance and/or replication (Fig. 1.1). Secondly, the 50 kD MAF appears to initiate the selective deactivation of primed respiratory burst responses of activated macrophages, and thus, may limit the duration of toxic oxygen intermediates generated by activated phagocytes. This effect would minimize unnecessary tissue damage incurred by activated macrophages at sites of inflammation, in an otherwise futile attempt to destroy reactive oxygen resistant pathogens. Thirdly, the 50 kD MAF induces potent nitric oxide production in goldfish macrophages. It is these macrophage-activating effects that lead me to hypothesize that the 50 kD MAF may be responsible for initiating the sequential induction of antimicrobial responses in goldfish macrophages.

The findings presented in this chapter also support a hypothesis that R3-type macrophages (i.e putative monocyte) may initiate the sequential induction of teleost macrophage antimicrobial mechanisms. In particular, it appears that the 50 kD MAF induces R3-type macrophages to initiate de-priming of macrophage respiratory burst responses. This conclusion is based on the following observations. Firstly, R3-type macrophages undergo de-priming of the respiratory burst response when stimulated with crude MAF preparations (see Fig. 4.8). The 30 kD MAF is not responsible for this de-priming effect, since IVDKM cultures stimulated with the 30 kD MAF continued to increase respiratory burst activity with increasing stimulation periods. It is only when the 50 kD MAF is used as a co-stimulus with the 30 kD MAF that IVDKM cultures undergo de-priming of the respiratory burst response (see Fig. 6.8). Secondly, R2-type macrophages do not appear to be responsible for de-priming macrophage respiratory burst activity since these cells continue to increase their respiratory burst potential with increased MAF stimulation

periods (i.e. preparations containing both the 30 kD and 50 kD MAFs, see Fig. 5.4). The kinetics of this response are similar to those observed in IVDKM stimulated with the 30 kD MAF only (see Fig. 6.8), and, at first glance, it would appear that R2-type macrophages may be unresponsive to the 50 kD MAF. However, R2-type macrophages produce nitric oxide in response to stimulation with crude MAF (Fig. 4.8), an antimicrobial response induced by the 50 kD MAF. Thus, although R2-type macrophages appear to respond to both the 30 kD and 50 kD MAFs, they are incapable of de-priming their respiratory burst response. It is my hypothesis that the 50 kD MAF initiates R3-macrophage-mediated de-priming of primed respiratory burst responses, and is responsible for the induction of sequential antimicrobial responses observed in GMCL and IVDKM cultures. The testing of this hypothesis will require that the kinetics of activation be determined in sorted macrophage sub-populations stimulated with purified MAFs. In addition, the kinetics of activation must also be determined in manipulated mixed sub-populations of macrophages (i.e. a certain number of R2-type macrophages mixed with a certain number of R3-type macrophages), stimulated with purified MAFs.

A novel observation of my study is the identification of a macrophage-deactivating factor present in crude MAF preparations. A 12 kD molecule, purified by GP-FPLC, inhibited nitric oxide production by goldfish macrophages co-stimulated with the 50 kD MAF and LPS . This is the first report of a macrophage-deactivating factor that regulates nitric oxide production in teleosts. Its presence in crude MAF preparations would explain why, on occasion, goldfish macrophages have reduced nitric oxide output, and could account for the individual variation of nitric oxide-inducing potentials obtained using various crude MAF preparations (see Figs. 3.5 and 6.3). The presence of this deactivating factor does not provide a complete explanation for the de-priming phenomenon

observed in my study. The stimulation of IVDKM with the partially purified 30 kD MAF and the purified 50 kD MAF was sufficient for induction of de-priming.

The de-priming of macrophage respiratory burst responses by the 50 kd MAF does not represent a classical form of deactivation of macrophage antimicrobial responses (i.e. a response that is induced by a deactivator). Instead, it appears to be a unique form of deactivation, in that select antimicrobial responses can be deactivated by the very factors that initially activate the macrophage to kill. In essence, macrophage activation *in vivo* may represent a pre-programmed "all-or-none" killing response that inevitably results in the sequential activation and deactivation of macrophage antimicrobial responses. The specific role that a macrophage-deactivating cytokine plays in this all-or-none killing response may be to inhibit the initial induction of macrophage activation (i.e. pre-treatment with deactivating cytokines may inhibit the actions of activating cytokines), or to dampen the overall production of toxic molecules generated by killer macrophages.

Table 6.1. Induction of nitric oxide production by R3-type macrophages stimulated with the 50 kD MAF partially purified by GP-FPLC.[a]

Treatment	μM Nitrite (± SEM)
control	-0.639 ± 0.191
MAF	-0.502 ± 0.171
LPS	0.218 ± 0.091
MAF + LPS	0.287 ± 0.069

[a] R3-type macrophages were sorted using flow cytometry and seeded into wells of a half area 96 well culture plate (2.5x104 cells/well). Cells were stimulated with the 50 kD MAF purified by GP-FPLC (1:4 dilution) and/or LPS (1 μg/ml). Nitrite content within the wells was determined 48 hrs later using the Griess reagents. The data presented represents the mean ± SEM of triplicate cultures.

Table 6.2. A summary of the various macrophage modulators that are present in crude cytokine preparations.

Molecule	Actions	Reference
50 kD MAF (two isotypes with varying isoelectric points)	• induces nitric oxide production in goldfish macrophages. • accelerates priming of the respiratory burst response of macrophages stimulated with the 30 kD MAF. • selectively deactivates primed macrophage respiratory burst response and initiates sequential induction of macrophage antimicrobial responses.	• this thesis
~30 kD MAF	• primes the respiratory burst response of goldfish and rainbow trout macrophages.	• this thesis • Graham and Secombes (1990b)
19 kD MAF	• primes the respiratory burst response of rainbow trout macrophages.	• Graham and Secombes (1990b)
12 kD deactivator	• inhibits nitric oxide production by goldfish macrophages.	• this thesis

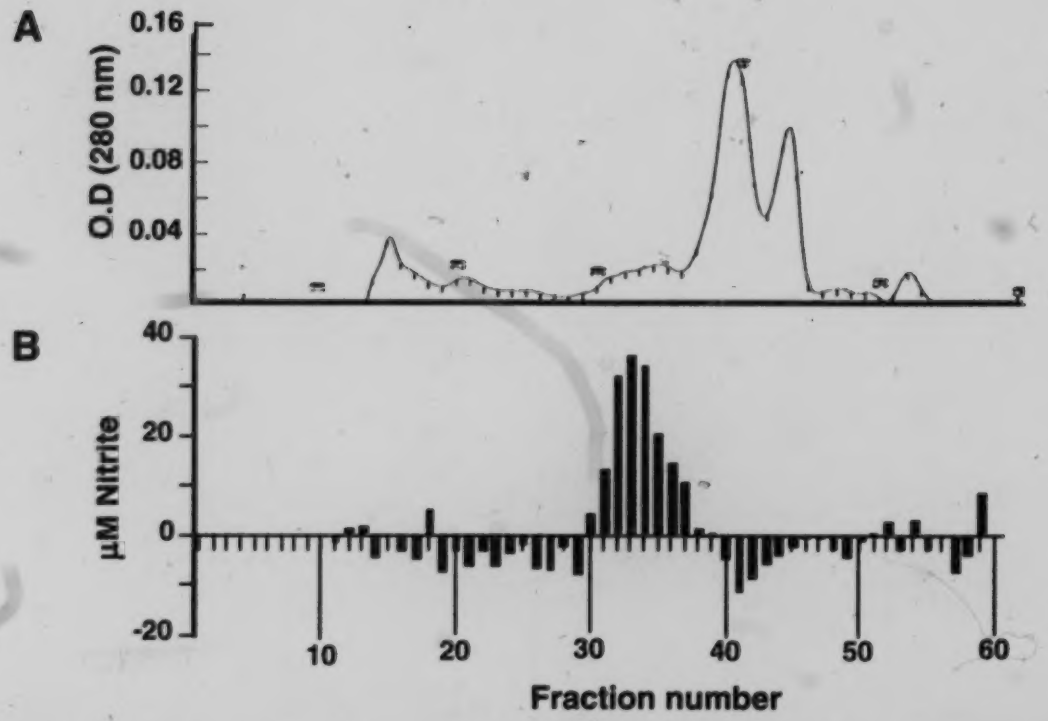

Figure 6.1. Production of nitric oxide by GMCL stimulated with GP-FPLC fractionated crude MAF. Crude MAF preparations were fractionated using a Superose 6 column (A), and MAF activity was determined in each fraction using the nitric oxide bioassay. GMCL were seeded into wells of a 96 well culture plate ($1x10^5$ cells/well) and stimulated with GP-FPLC fractioned crude MAF (1:4 dilution) and LPS (5 µg/ml). Nitrite production was determined 72 hrs later using the Griess reagents. Each bar represents the mean ± SEM of triplicate cultures. and is reprentative of two experiments that were performed.

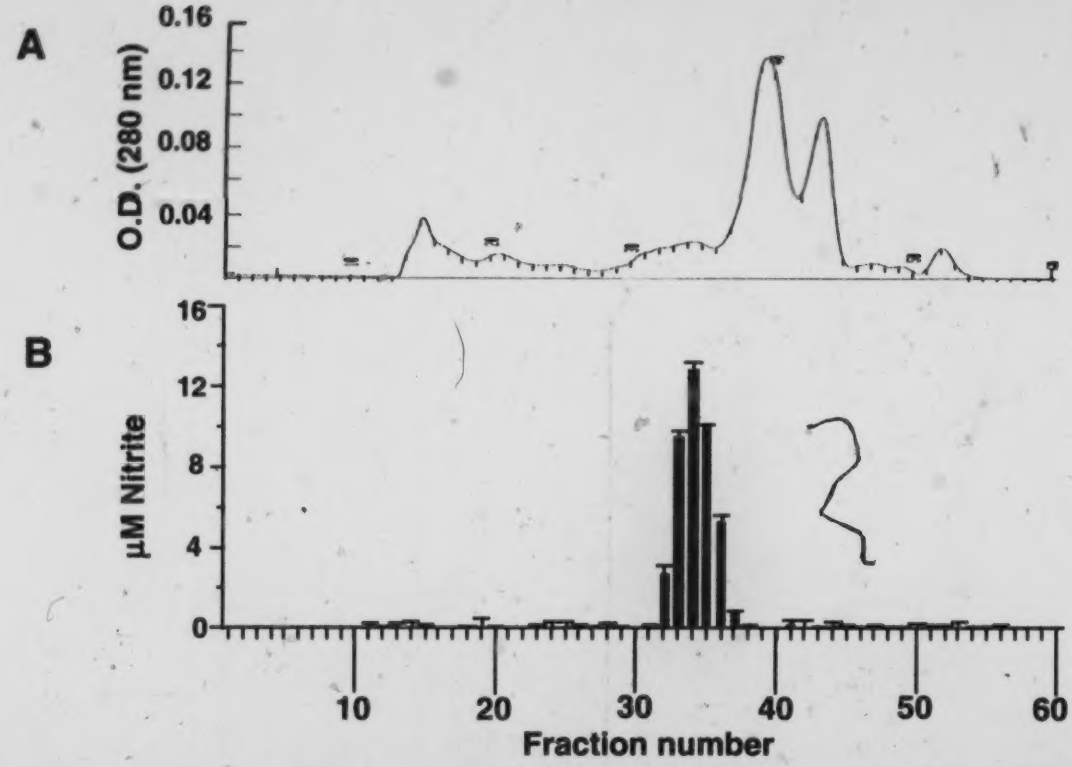

Figure 6.2. Production of nitric oxide by IVDKM stimulated with GP-FPLC fractionated crude MAF. Crude MAF preparations were fractionated using a Superose 6 column (A), and MAF activity was determined in each fraction using the nitric oxide bioassay (B). IVDKM were seeded into wells of a half area 96 well culture plate ($5x10^4$ cells/well) and stimulated with individual fractions from GP-FPLC fractionated crude MAF (1:4 dilution) and LPS (1µg/ml). Nitrite production was determined 72-hrs later using the Griess reagents. Each bar represents the mean ± SEM of triplicate cultures and is reprentative of three experiments that were performed.

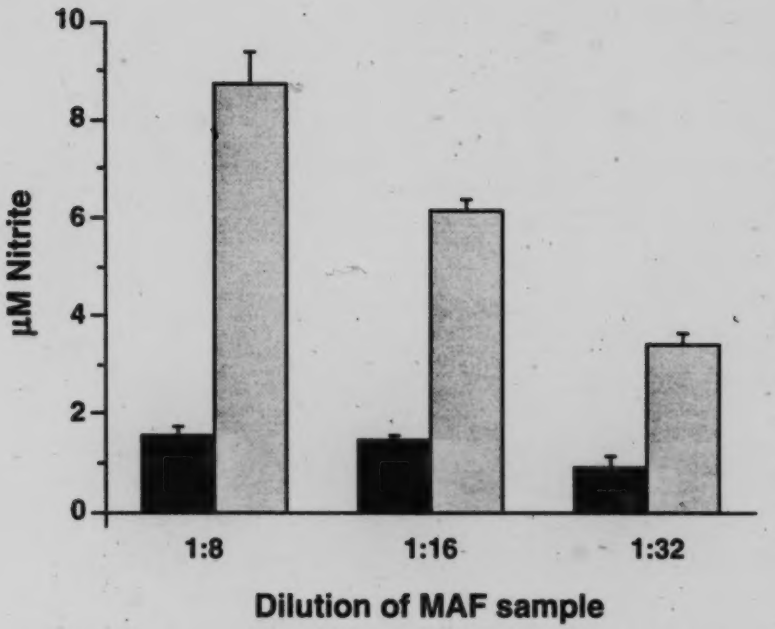

Figure 6.3 Comparison between the nitric oxide-inducing potential of a crude MAF preparation and the GP-FPLC partially purified 50 kD MAF. IVDKM^{f}were seeded into wells of a half area 96 well culture plate ($5x10^4$ cells/well) and stimulated with a crude MAF preparation (black) or the GP-FPLC partially purified 50 kD MAF, originally isolated from that same crude preparation. All cultures were also co-stimulated with LPS (1 µg/ml). Nitrite content was determined using the Griess reagents 72 hrs after stimulation. Each bar represent the mean ± SEM of triplicate cultures.

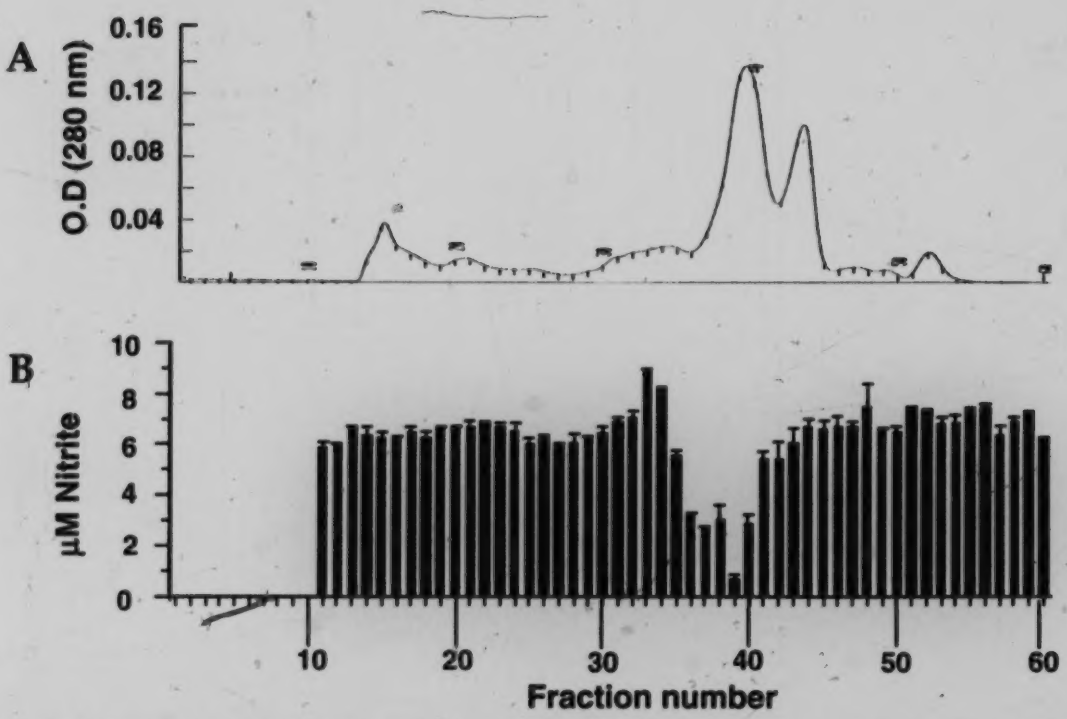

Figure 6.4. Inhibition of macrophage nitric oxide production by a deactivator present in crude MAF preparations. Crude MAF preparations were fractionated by GP-FPLC using a Superose 6 column (A) and macrophage deactivating activity determined using the nitric oxide assay (B). IVDKM were seeded into wells of a half area 96 well culture plate ($5x10^4$ cells/well) and pre-treated with individual fractions of GP-FPLC fractionated crude cytokine preparations for 6 hrs. IVDKM were subsequently activated in all groups with the GP-FPLC partially purified50 kD MAF (1:16 dilution) and LPS (1µg/ml). Nitrite production was determined 72 hrs later using the Griess reagents. Each bar represents the mean ± SEM of triplicate cultures and is reprentative of two experiments that were performed.

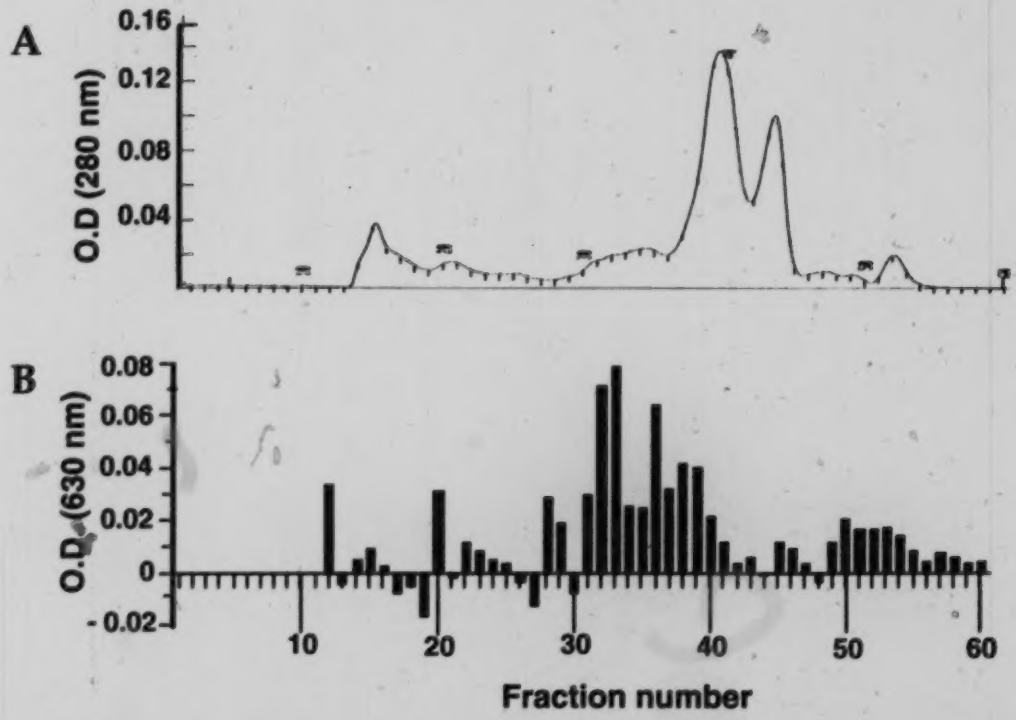

Figure 6.5. Priming of the respiratory burst response of IVDKM with GP-FPLC fractionated crude MAF. Crude MAF preparations were fractionated using a Superose 6 column (A), and MAF activity determined using the respiratory burst assay (B). IVDKM were seeded into wells of a half area 96 well culture plate ($5x10^4$ cells/well) and stimulated with individual fractions of GP-FPLC fractionated crude MAF (1:3 dilution). Respiratory burst responses were measured 6 hrs later using the NBT assay. Each bar represents the mean ± SEM of triplicate cultures and is reprentative of two experiments that were performed.

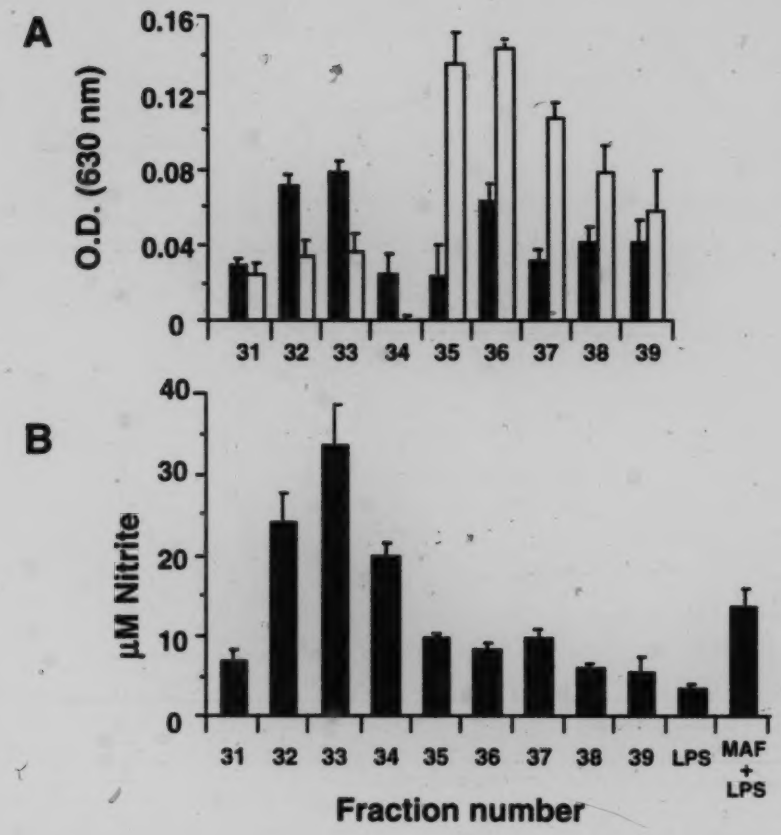

Figure 6.6. Effect of incubation period on priming goldfish macrophage respiratory burst response by fractions obtained from GP-FPLC fractionated crude MAF. GMCL were seeded into wells of a 96 well culture plates ($1x10^5$ cells/well) and stimulated with a 1:3 dilution of fractions 31-39 of GP-FPLC fractionated crude MAF. IVDKM respiratory burst responses were determined using the NBT assay 6 hrs (black) or 24 hrs (white) after stimulation. Note the two distinct activating activities in fractions 33 (50 kD MAF) and 36 (30 kD MAF). Also illustrated is the relative nitric oxide-inducing potential of each of these fractions (B). Each bar represents the mean ± SEM of triplicate cultures and are representative of two experiments that were performed.

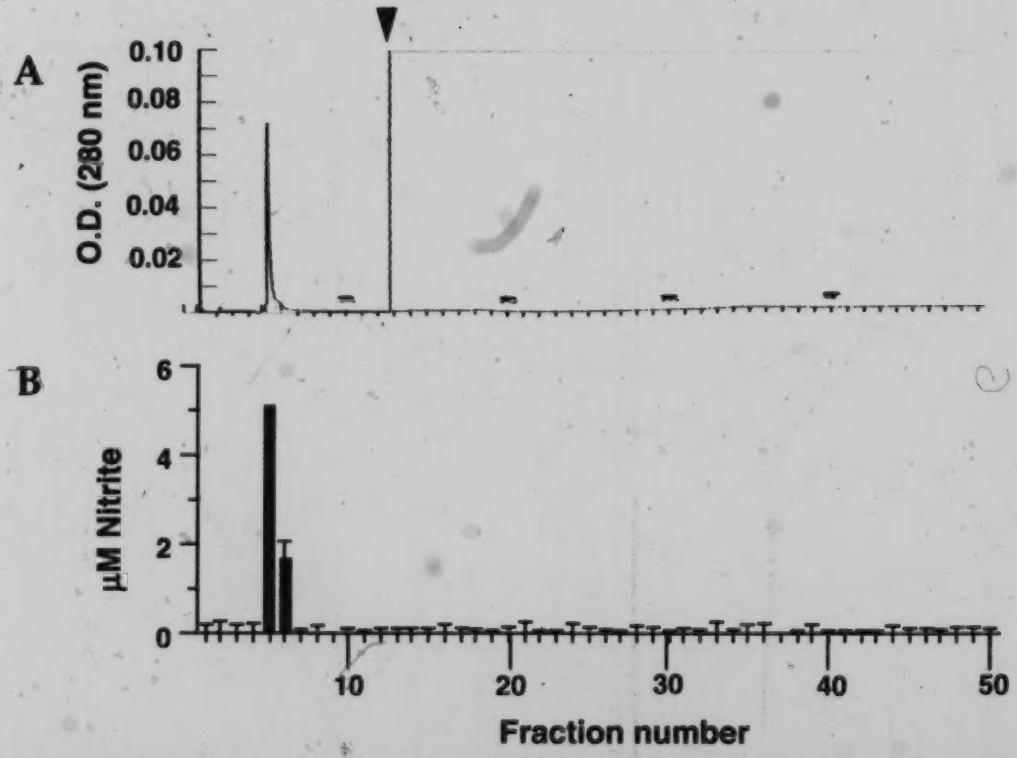

Figure 6.7. Nitric oxide-inducing activity of the 50 kD MAF (partially purified using GP-FPLC) after fractionation by C-FPLC. C-FPLC was done using a pH elution gradient of 7.5 - 5.0. A typiçal chromatogram is illustrated in "A" (arrow indicates initiation of the descending pH gradient). Nitric oxide inducing activity of the purified samples was done by stimulating IVDKM ($5x10^4$ cells/well) with a 1:4 dilution of C-FPLC fractionated samples and LPS (1 µg/ml). Nitrite content was determined 48 hrs later using the Griess reagents (B). Each bar represents the mean ± SEM of triplicate cultures and are representative out of two experiments that were performed.

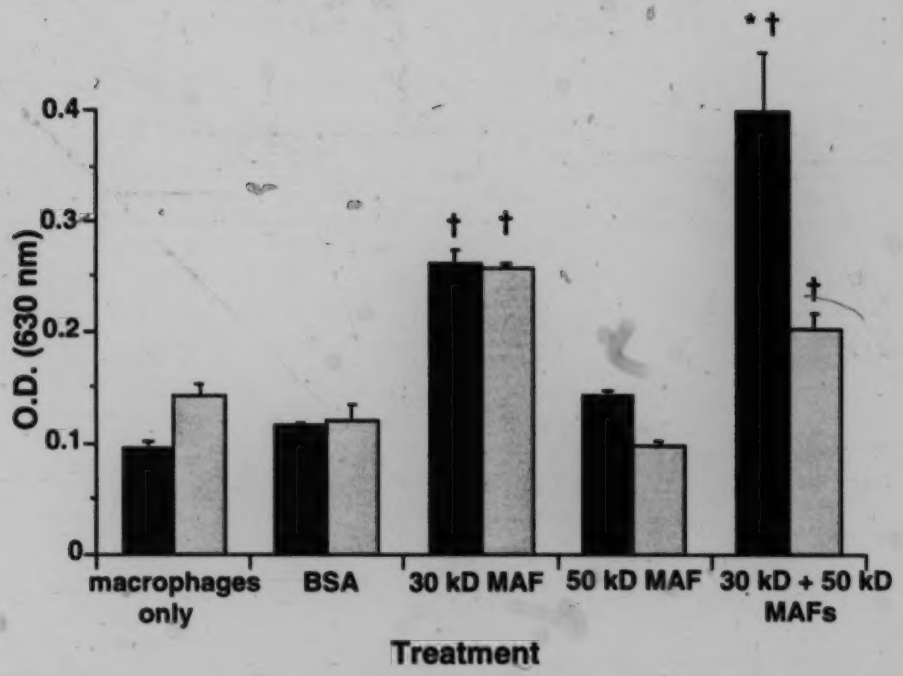

Figure 6.8. Kinetics of priming the respiratory burst response of IVDKM by the 30 kD and 50 kD MAFs. IVDKM were seeded in half area 96 well culture plates ($1x10^5$ cells/well) and stimulated with a 1:4 dilution of the 30 kD MAF (partially purified by GP-FPLC) and/or a 1:4 dilution of the 50 kD MAF (purified by GP-FPLC and C-FPLC). As an additional control, IVDKM were also stimulated with BSA (10 µg/ml) in order to demonstrate the specific priming action of these purified MAFs. Respiratory burst responses were measured using the NBT assay 18 hrs (black) or 36 hrs (gray) after stimulation. Each bar represents the mean + SEM of five cultures. (†) $P < 0.05$, comparing O.D. values between treated and control macrophages at the same time point. (*) $P < 0.05$, comparing O.D. values at 18 hrs and 36 hrs within the same treatment group.

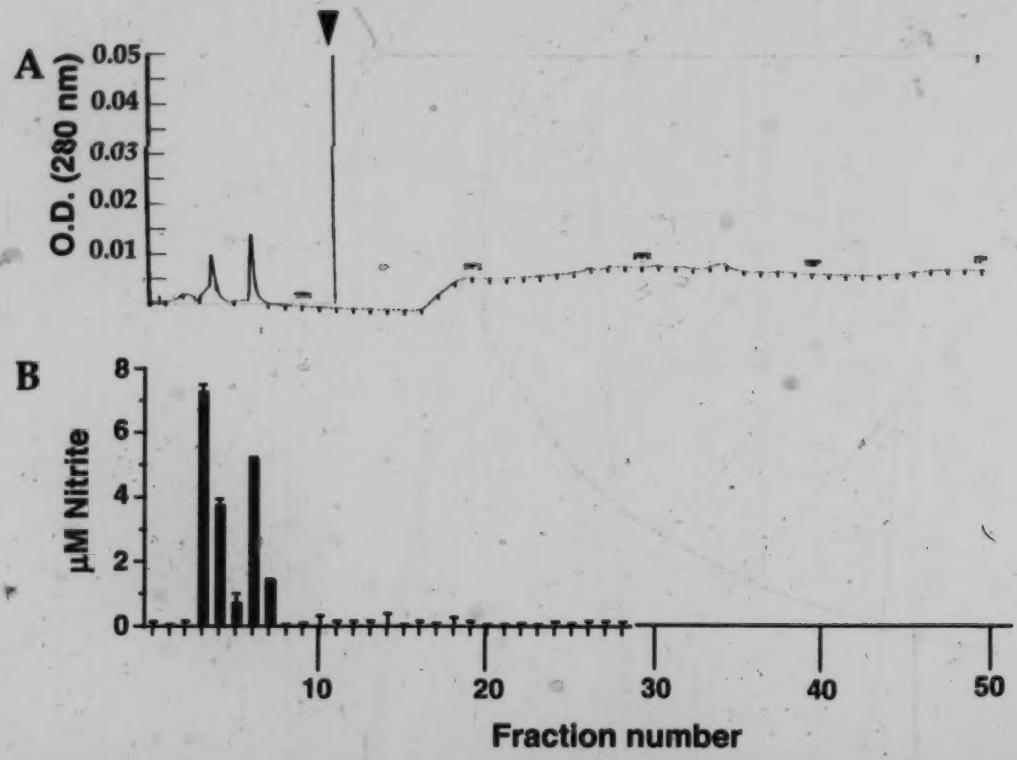

Figure 6.9. Nitric oxide-inducing activity of the 50 kD MAF (purified using GP-FPLC) after fractionation by C-FPLC. C-FPLC was done using a pH elution gradient of 9.3 - 6.0. A typical chromatogram is illustrated in "A" (arrow indicates initiation of the descending pH gradient). Nitric oxide inducing activity of the purified samples was done by stimulating IVDKM ($5x10^4$ cells/well) with a 1:4 dilution of C-FPLC fractionated samples and LPS (1 µg/ml). Nitrite content was determined 48 hrs later using the Griess reagents (B). Each data point represents the mean ± SEM of triplicate cultures and are representative of two experiments that were performed.

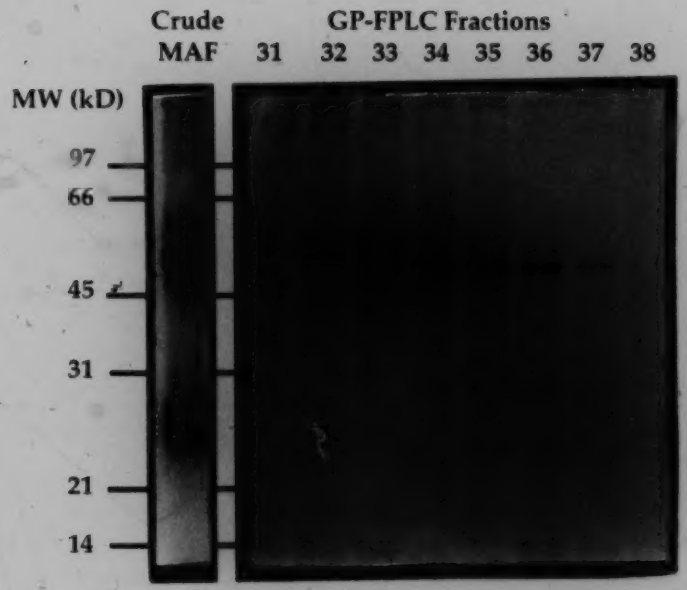

Fig 6.10. SDS-PAGE analysis of nitric oxide-inducing fractions collected from GP-FPLC fractionated crude MAF. Ten µl aliquots from the GP-FPLC fractions listed were electrophoresed through 5% stacked, 7.5-15% SDS-PAGE gels under non-reducing conditions, and proteins subsequently visualized by staining the gel with silver. The nitric oxide-inducing capacity of these fractions was presented in Figs 6.1 and 6.2.

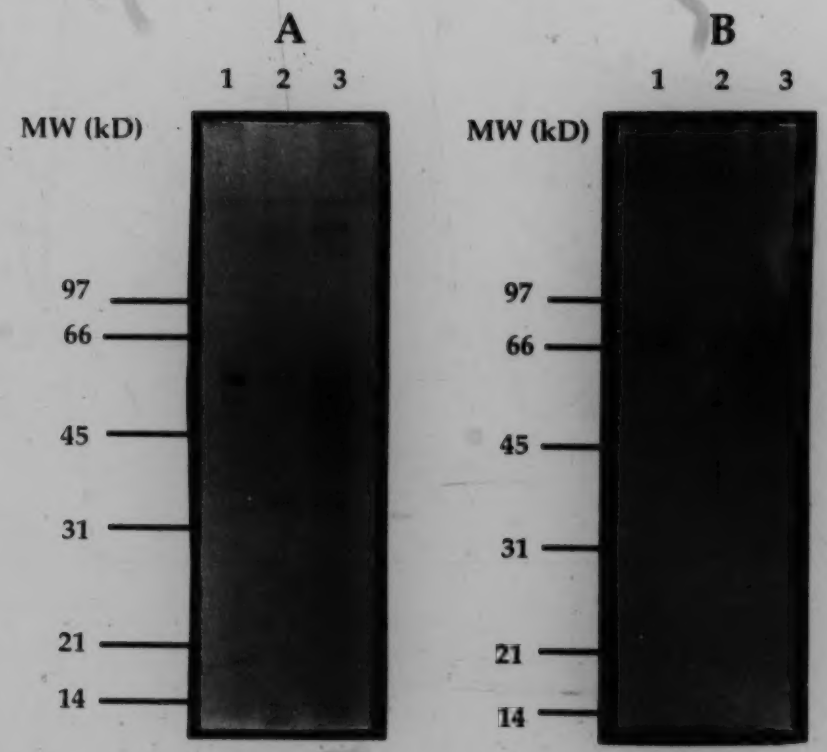

Fig 6.11. SDS-PAGE analysis of a nitric oxide-inducing MAF purified from crude cytokine supernatants using GP-FPLC and C-FPLC. Crude cytokine preparations were first fractionated using GP-FPLC, and fractions inducing maximal nitric oxide production in IVDKM (fractions 33 and 34, Fig 6.2) were pooled. These samples were subsequently fractionated using C-FPLC (pH gradient, 9.3 - 6.0). Two nitric oxide inducing activities were obtained using C-FPLC (fractions 4 and 7, Fig. 6.9). Ten µl aliquots from GP-FPLC (lane 3) and C-FPLC purified MAF (lane 1= fraction 7, lane 2= fraction 4) were electrophoresed through 5% stacked, 7.5-15% SDS-PAGE gels under non-reducing (panel A) or reducing conditions (panel B). Proteins were visualized by staining the gels with silver.

CHAPTER VII

GENERAL DISCUSSION

Overview of the findings

Macrophages are phagocytic leukocytes, and are essential effector cells in host defense against pathogens. These normally quiescent cells can be transformed into potent killer cells upon stimulation with cytokines and/or pathogen-derived molecules; a phenomenon known as macrophage activation. Macrophages possess a repertoire of inducible antimicrobial mechanisms that are very effective in killing pathogenic microorganisms. Activated macrophages produce a number of highly toxic molecules, through a series of highly regulated pathways that are necessary to prevent tissue destruction at sites of inflammation.

By comparison to mammals, little is known about the molecular mechanisms mediating macrophage activation in teleosts. Initial studies, carried out using rainbow trout macrophages, clearly demonstrated the importance of cytokines in mediating the induction of macrophage activation in teleosts (Chung and Secombes, 1987; Graham and Secombes, 1988; 1990 a,b). Most of the work on the function of fish macrophages has been limited to studies directed at elucidating the mechanisms leading to cytokine induction of the macrophage respiratory burst response. Moreover, the characterization of cytokines involved in the activation of respiratory burst activity has been done using crude cytokine preparations that contain MAF activity. The biochemical nature of these cytokines remains to be elucidated.

Objectives of the thesis

The objectives of my thesis were to characterize inducible antimicrobial responses of cytokine-activated teleost macrophages and to

determine the role that host-derived and pathogen-derived molecules play in the induction of macrophage activation in goldfish. The elucidation of the molecular mechanisms involved in the induction of macrophage activation in fish has been hindered by the inability to obtain large numbers of highly purified macrophages from fish. Until recently, there were no established teleost macrophage cell lines, and most of the work in this area was dependent on the isolation of macrophage sub-populations from specific tissues.

Characterization of goldfish macrophages

Our laboratory has recently established a spontaneously proliferating goldfish macrophage cell line that shows morphological, cytochemical and functional similarities to goldfish kidney macrophages (Wang et al., 1995). This cell line was an invaluable asset for identifying of the molecular mechanisms mediating the induction of macrophage activation in goldfish. However, since cellular responses elicited by cell lines may not necessarily represent important functional responses *in vivo*, I designed an *in vitro* cultivation technique to obtain sufficient numbers of primary macrophage for my experiments. The technique I developed was similar to what is commonly done for obtaining large numbers of macrophages from mice; where the hematopoeitic tissues of the host are used as a source of macrophage progenitor cells and are induced to proliferate by stimulation with supernatants containing MGF (Metcalf et al., 1967; Neumann and Sorg, 1980; Belosevic et al., 1988). In my experiments, the hematopoeitic kidney tissues of goldfish acted as a source of macrophage progenitor cells. Cells derived from goldfish kidney leukocyte cultures grown in the presence of CCM were characterized as macrophages, based on cytochemistry, morphology, function, and flow cytometric profile. Interestingly, these IVDKM cultures contained three distinct sub-populations of macrophages, which were designated R1, R2 and R3-type macrophages. These three sub-populations of macrophages

were also present in GMCL, and appear to represent cells arrested at distinct differentiation junctures in macrophage development.

The morphology of R1-type macrophages suggested that these cells may be similar to mammalian monoblasts (Beelen et al., 1989; Stachura, 1989). R1-type macrophages had high proliferative potentials, increasing in number almost four fold over 8 days of cultivation. This sub-population of macrophages proliferated in the absence of exogenously added MGF, albeit at a slower rate than those cultured in the presence of MGF. Furthermore, proliferating cells appeared to differentiate into R2-type macrophages.

R2-type macrophages were morphologically and cytochemically similar to mature tissue macrophages; having abundant cytoplasm, irregular shape, and extensive vacuolization. R2-type macrophages developed from the differentiation of both R1 and R3-type macrophages. In addition to their development from R1 and R3-type macrophages, R2-type macrophages also self-replicated. Functionally, R2-type macrophages were highly phagocytic, and produced reactive oxygen and nitrogen intermediates when stimulated with cytokines and/or LPS.

R3-type macrophages appeared to be a temporally arrested macrophage sub-population in IVDKM cultures, and were morphologically, cytochemically, and functionally similar to monocytes. R3-type macrophages were dependent upon the presence of CCM for sustained viability, and were incapable of proliferation, even in the presence of exogenously added MGF. This sub-population of macrophage differentiated into R2-type macrophages when cultured in the presence of CCM, MAF, or LPS. The differentiation of R3->R2-type macrophages in response to stimulation with LPS only, suggests that R3-type macrophages are autocrine producers of their own differentiation factor(s). Although R3-type macrophages are potent producers of reactive oxygen, they produce only small quantities of nitric oxide.

In mammals, hematopoeitic-derived macrophages go through the following sequence of development: monoblast -> promonocyte -> monocyte -> macrophage. My data demonstrate that macrophage development in fish, although capable of following a similar pattern, may also proceed from a putative monoblastic stage (R1-type macrophage) to a mature macrophage (R2-type macrophage) without going through the intermediate monocyte-like stage of development (R3-type macrophage). Evidence for this pattern of development was obtained by demonstrating that R1-type macrophages developed into R2-type macrophages without the concomitant development of an R3-type macrophage sub-population. There is evidence suggesting that mammalian macrophages can arise irrespective of a monocyte stage. Microglia within the central nervous system were shown to develop from macrophage progenitor cells found in the central nervous system, and thus re-population of microglia may not be dependent upon influx of monocytes from the circulation (Alliot et al., 1991). Moreover, the elimination of circulating monocytes by strontium treatment does not affect the proliferative potential of resident peritoneal macrophages, alveolar macrophages, or Kupffer cells (Sawyer et al., 1982; Shibata et al., 1985; and Yamamda et al., 1990). Thus, it is possible that multiple pathways of macrophage development occur within IVDKM cultures: 1) through normal hematopoeitic pathways, 2) through immediate progenitor development, and 3) by self-replication.

Mechanisms regulating the induction of goldfish macrophage antimicrobial functions

Regulation of nitric oxide production

Our laboratory was the first to demonstrate that goldfish macrophages synthesized nitric oxide in response to stimulation with bacterial LPS. In this thesis, I demonstrated that crude MAF preparations, produced from the mitogenic stimulation of goldfish kidney leukocytes, acted as a synergistic signal for induction of nitric oxide synthesis in LPS-

stimulated goldfish macrophages (Neumann et al., 1995; this thesis). More specifically, R2-type macrophages were responsible for the production of nitric oxide in both GMCL and IVDKM cultures. R2-type macrophages displayed a prototypical macrophage response when stimulated with MAF and/or LPS, producing no nitric oxide when stimulated with either factor, but copious amounts when co-stimulated with both factors. In contrast, R3-type macrophages (putative monocyte) produced little or no nitric oxide in response to stimulation with MAF and/or LPS. This observation is consistent with that reported for human monocytes and caprine monocyte-derived macrophages (Murray and Teitelbaum, 1992; Schneemann et al., 1993; Adler et al., 1996). Recent experiments have shown that human monocytes acquire the potential to produce nitric oxide after maturation (Martin and Edwards, 1993; Bose and Farnia, 1995). Thus, it seems plausible that R3-type macrophages may acquire the potential to produce nitric oxide after differentiation into R2-type macrophages.

Goldfish macrophages also produced nitric oxide when infected with the Gram positive bacteria, *Mycobacterium chelonei*, or the obligate intracellular protozoan pathogen of mammalian macrophages, *L. major*. MAF enhanced production of nitric oxide by goldfish macrophages infected with either pathogen. Interestingly, goldfish macrophages produced nitric oxide in response to intracellular infection only. Both IVDKM and GMCL secreted large amounts of nitric oxide when infected with *L. major*. These data contrasted with those observed using a mouse macrophage cell line infected with *L. major*. Mouse macrophages required IFN-γ as an accessory signal for induction of nitric oxide synthesis, an observation consistent with that reported by others (Green et al., 1990, 1991; Guy and Belosevic, 1993). The induction of nitric oxide in response to intracellular infection was not a result of phagocytosis by goldfish macrophages, since latex beads did not induce nitric oxide production. Thus, the formation of nitric oxide in goldfish macrophages is induced

following the recognition of a pathogen-derived molecule(s). Their ability to produce nitric oxide in response to intracellular infection may represent a more primitive regulatory pathway for the induction of this antimicrobial response in fish. In mammals, macrophage-derived nitric oxide production appears to become more tightly regulated, requiring both the recognition of pathogen-derived molecules and cytokine stimulation for induction.

Nitric oxide production in goldfish macrophages, like in mammalian macrophages, is L-arginine dependent, and can be inhibited by culturing macrophages in the presence of L-arginine analogs, such as N^GMMLA and amino guanidine (Wang et al., 1995; Neumann et al., 1995). The inducible form of teleost macrophage nitric oxide synthase has been confirmed at the nucleotide sequence level by Hardie et al. (1994) and Laing et al. (1996), who have recently reported a partial nucleotide sequence from rainbow trout and goldfish macrophages, respectively. The partial nucleotide sequences between these two diverse teleost species shows 87% homology, and is approximately 70% homologous to mammalian macrophage iNOS sequences.

Regulation of the respiratory burst

The respiratory burst is another antimicrobial mechanism of activated teleost macrophages. Macrophages can be primed for enhanced respiratory burst activity upon stimulation with cytokines and/or LPS. I have demonstrated that the respiratory burst of goldfish macrophages can be rapidly primed after stimulation with MAF (i.e. 6 hrs). This primed response plateaued during the next 6 hrs and was subsequently deactivated, such that 24-48 hrs after stimulation the respiratory burst of MAF-activated macrophages was similar to that of unstimulated macrophages. In contrast, LPS enhanced the macrophage respiratory burst response with increased duration of stimulation. Interestingly, MAF and LPS co-stimulated macrophages followed the kinetics of priming similar

to that induced by MAF only. The priming of macrophages was significantly greater in co-stimulated cells than in MAF-only treated macrophages during the first 12 hrs of stimulation. Thus, LPS augmented MAF-induced priming of the respiratory burst early after stimulation, but MAF antagonized LPS-induced priming after extensive stimulation periods (> 24 hrs).

The results discussed above represent the kinetics of priming induced by MAF and/or LPS in IVDKM and GMCL. The various macrophage sub-populations comprising IVDKM demonstrated unique kinetics of priming in response to stimulation with MAF and/or LPS. R2-type macrophages increased respiratory burst potential in response to longer stimulation periods with MAF and/or LPS. In contrast, R3-type macrophages rapidly increased their respiratory burst potential in response to stimulation with MAF and/or LPS (i.e. 6 hrs), but subsequently underwent deactivation of their primed state. Neither R2 nor R3-type macrophages completely mimicked the kinetics of priming observed for activated IVDKM or GMCL cultures. Stimulation of IVDKM or GMCL with LPS resulted in increased respiratory burst potentials with increasing cultivation periods, a characteristic shared by R2-type macrophages. However, whenever MAF was used as a stimulus, IVDKM and GMCL displayed the kinetics of priming similar to that of R3-type macrophages. It appears that the macrophage sub-populations present in IVDKM and GMCL may actively cross-regulate each other's functional activities *in vitro*. The ability of a specific macrophage sub-population to regulate the respiratory burst of another macrophage sub-population is a novel concept in macrophage biology.

It is interesting that the mature R2-type goldfish macrophage displays the characteristic kinetics of MAF-induced priming that has been described by others. Rainbow trout and dab resident kidney macrophages require extensive incubations with MAF (i.e. 48 hrs) for priming of their respiratory burst potentials (Chung and Secombes, 1987; Graham and

Secombes, 1988; Graham and Secombes, 1990a, 1990b; Hardie et al., 1994; Francis and Ellis, 1994; Jang et al., 1995; Tahir and Secombes, 1996).

There is evidence that mammalian macrophages, under specific circumstances, can be induced to undergo rapid priming of their respiratory burst response. Murine macrophages can undergo rapid priming of their respiratory burst in response to stimulation with LPS or other pathogen-derived molecules (Lin et al., 1993). Moreover, human monocyte respiratory burst potential can be rapidly primed (i.e. 30 min) in response to stimulation with IFN-γ or TNF-α (Kumaratilake et al., 1990). Our laboratory has also demonstrated that murine bone marrow-derived macrophages undergo rapid priming of their respiratory burst when stimulated with IFN-γ and LPS (Fig. 7.1). Taken together, these observations suggest that the regulation of respiratory burst responses in vertebrate macrophages, by cytokines and pathogen-derived molecules, may be similar under certain circumstances.

The production of reactive oxygen and nitrogen intermediates was biphasic in goldfish macrophages. These cells were primed for respiratory burst activity early after stimulation with MAF and LPS (i.e. 6 hrs), but subsequently underwent deactivation of their primed state. This deactivation event did not represent an overall deactivation of macrophage antimicrobial mechanisms, since macrophages retained the ability to produce nitric oxide. My results support a hypothesis that teleost macrophages undergo a sequential induction of temporally spaced antimicrobial mechanisms. The importance of this phenomenon in host defense is discussed below in detail.

Characterization of macrophage activating factors

The kinetics of MAF-induced nitric oxide and respiratory burst activity in goldfish macrophages was shown to be mediated by a number of modulators present in crude MAF preparations. Both macrophage

activating and deactivating molecules were identified in these crude cytokine preparations using FPLC.

The 30 kD MAF

A 30 kD MAF, partially purified from crude cytokine preparations using GP-FPLC, primed macrophage respiratory burst responses, but induced only minor production of nitric oxide. The kinetics of priming induced by the 30 kD MAF were similar to those observed when goldfish macrophages were stimulated with LPS only, suggesting that this 30 kD molecule may be a MAF produced by kidney leukocytes in response to stimulation with LPS. It is possible that this molecule is equivalent to mammalian TNF-α. Human recombinant TNF-α has been shown to prime rainbow trout macrophage respiratory burst response, and the addition of soluble human recombinant TNF-α receptors to crude MAF preparations impairs the ability of these crude preparations to prime the respiratory burst (Jang et al., 1995; Tahir and Secombes, 1996; Campos-Perez et al., 1996). My observation, that a 30 kD MAF induced priming of goldfish macrophage respiratory burst activity, is consistent with that observed by Graham and Secombes (1990a), who demonstrated that a 32 kD MAF, partially purified from crude cytokine preparations using gel permeation chromatography, induced priming of the respiratory burst response of rainbow trout resident kidney macrophages.

The 50 kD MAF

A 50 kD MAF, purified from crude cytokine preparations using GP-FPLC and C-FPLC, was shown to induce nitric oxide production in goldfish macrophages. Moreover, this molecule rapidly enhanced priming of the respiratory burst response of macrophages stimulated with the 30 kD MAF. However, the 50 kD MAF subsequently antagonized the priming effect of the 30 kD MAF after long-term stimulation (i.e. 48 hrs), and thus

appeared to be responsible for the sequential induction of goldfish macrophage antimicrobial responses.

The kinetics of this response was identical to that observed for IVDKM or GMCL stimulated with MAF and LPS, suggesting that the 50 kD MAF may be responsible for deactivation primed goldfish macrophage respiratory burst activity. The deactivation of the macrophage respiratory burst response by the 50 kD MAF does not represent a classical deactivation of macrophage antimicrobial responses (i.e. that it is induced by a deactivator), since this molecule has the ability to activate macrophages to produce nitric oxide. Instead, this deactivation response represents a unique form of deactivation, in that select antimicrobial responses can be deactivated by the very factors that initially activate the macrophage to kill.

The 50 kD MAF was fractionated into two distinct nitric oxide-inducing activities, using basic gradients in C-FPLC (pH 6.0 - 9.3). One molecule had a pI close to 9.3, whereas the other was greater than 9.3. SDS-PAGE revealed that these two activities contained almost identical proteins. Under non-reducing conditions the more basic 50 kD MAF was comprised of two doublets; a 59 kD/ 55 kD doublet, and a 35 kD/ 33 kD doublet. The less basic 50 kD MAF was comprised of a single doublet (59 kD/ 55 kD) and a single band corresponding to 33 kD. Thus, these two MAFs differed only by the absence of the 35 kD band in the less basic form. However, under reducing conditions, a single doublet of 72 kD/ 67 kD was observed from both of these MAFs.

My findings implicate several fundamentally important roles of the 50 kD MAF in host defense. First, and foremost, this molecule rapidly activates macrophages for enhanced killing, and appears to accelerate cytokine-induced priming of the respiratory burst response of goldfish macrophages. The ability of macrophages to respond rapidly to intracellular infection is crucial for preventing dissemination of disease, particularly diseases caused by pathogens that utilize macrophages as host

cells (see Figure 1.1). Moreover, the 50 kD MAF appears to initiate the selective deactivation of primed respiratory burst responses of activated macrophages, as determined by its obligate presence for deactivation. Thus, in addition to its ability to rapidly induce priming of the respiratory burst of goldfish macrophages, the 50 kD MAF also initiates the deactivation of this response, limiting the duration of production of toxic oxygen intermediates by activated phagocytes. Finally, the 50 kD MAF induces potent nitric oxide production in goldfish macrophages. Taken together, my results suggest that the 50 kD MAF is a central regulatory molecule that initiates the induction of temporally spaced antimicrobial mechanisms of goldfish macrophages.

The 12 kD macrophage deactivator

Crude MAF preparations also contained a macrophage deactivating factor. A 12 kD molecule, purified by GP-FPLC, inhibited production of nitric oxide by cytokine and LPS-activated goldfish macrophages. This is the first report of a macrophage deactivating factor that regulates nitric oxide production in fish. Its presence in crude MAF preparations would explain why, on occasion, goldfish macrophages had reduced nitric oxide output, and could account for the individual variation of nitric oxide-inducing activity observed between various crude MAF preparations.

A model of macrophage activation in vertebrates

The data presented in my thesis describe a novel phenomenon of macrophage activation: the sequential induction and deactivation of antimicrobial responses of goldfish macrophages. Our current concept of macrophage activation, based on the activation kinetics observed in mammalian macrophages, suggests that these responses are simultaneously induced. However, there is evidence in the literature that

supports the hypothesis that vertebrate macrophages, in general, may mount sequential antimicrobial responses. I developed a model, shown in Fig. 7.2, that describes the kinetics of the sequential induction of vertebrate macrophage antimicrobial responses. The basis of this model finds its support in the mechanistic coupling of the inducible antimicrobial responses of mammalian macrophages (i.e. the respiratory burst, tryptophan degradation, and nitric oxide production).

Although the literature suggests that the respiratory burst and nitric oxide production represent two independent macrophage killing mechanisms (i.e. the induction of one does not depend on the induction of the other, Iyengar et al., 1987), both responses can be linked to the degradation of tryptophan. IDO, an IFN and LPS-inducible enzyme, uses superoxide as the substrate for cleaving the indoleamine ring of tryptophan (Hayashi et al., 1977). Superoxide is the immediate enzymatic product generated by the respiratory burst oxidase (Berton et al., 1988; Borregaard, 1988). Induction of the respiratory burst prior to tryptophan degradation would ensure sufficient quantities of superoxide as a substrate for IDO activity. In addition, metabolites of tryptophan degradation are potent scavengers of reactive oxygen, acting as molecular sinks for reactive oxygen intermediates under hyperoxia conditions (Christen et al., 1990). The induction of tryptophan degradation simultaneously with the respiratory burst would result in an extremely inefficient killing response, since reactive oxygen would be actively scavenged by tryptophan catabolites. Off-setting the induction of these two cytotoxic responses, as seen in Fig 7.1, would maximize the killing effects of both the respiratory burst and tryptophan degradation. Superoxide generated during the respiratory burst would initially be used for the generation of reactive oxygen intermediates, and thereafter as a substrate for the degradation of tryptophan. In turn, catabolites of tryptophan degradation could detoxify reactive oxygen, providing a temporal regulation of reactive oxygen-

induced cytotoxicity, thus minimizing immunopathology induced by activated phagocytes at sites of inflammation.

The degradation of tryptophan is also coupled to production of nitric oxide by macrophages. Picolinic acid, a catabolite of tryptophan degradation, synergizes with IFN-γ for inducing nitric oxide production in murine macrophages (Melillo et al., 1993; 1996 a,b; 1997 a,b). Picolinic acid exerts its nitric oxide-inducing potential via a hypoxic responsive element located in the 5′ flanking region of the murine iNOS gene (Melillo et al., 1996a; 1997 a,b). Mutation or deletion of this promoter sequence impairs picolinic acid-induced gene transcription of iNOS without affecting induction of nitric oxide synthase by LPS (Melillo et al., 1996). Off-setting the induction of tryptophan degradation and nitric oxide production in macrophages would ensure sufficient quantities of picolinic acid for the synergistic induction of nitric oxide. Moreover, if the respiratory burst was simultaneously induced with nitric oxide production, then picolinic acid could not exert its nitric oxide-inducing effects, since the respiratory burst creates an extremely hyperoxic microenvironment. The induction of tryptophan degradation prior to nitric oxide induction would facilitate the generation of a hypoxic microenvironment, as a result of tryptophan catabolites actively scavenging reactive oxygen intermediates. This would permit picolinic acid to augment nitric oxide production by macrophages.

Nitric oxide appears to be the terminal killing mechanism of activated vertebrate macrophages. In addition to its potent killing effects (Table 1.3), nitric oxide is a deactivator of specific enzymes involved in macrophage cytotoxic reactions. Nitric oxide is an inhibitor of both protein kinase C and IDO, enzymes involved in the activation of the respiratory burst and tryptophan degradation, respectively (Gopalakrishna et al., 1993; Thomas et al., 1994). Moreover, nitric oxide acts as a negative feedback inhibitor of its own synthesis (Assreuy et al., 1993; Griscavage et al., 1993). The induction of nitric oxide simultaneously with the respiratory burst and tryptophan degradation would antagonize the killing

effects of these two latter responses. Off-setting the induction of nitric oxide after the respiratory burst and tryptophan degradation would ensure maximal killing of the latter two responses, as well as temporally regulate production of reactive oxygen and nitrogen intermediates by activated macrophages.

This sequential induction and deactivation of macrophage antimicrobial responses may represent an important functional response in host defense against pathogens. The highly cytotoxic nature of activated macrophages necessitates the need for strict regulatory control of this cellular process. As such, macrophage activation is controlled by both spatial and temporal regulatory mechanisms (Table 7.1). Sequential induction and deactivation of macrophage cytotoxic responses provides an additional form of temporal regulation of macrophage activation, limiting the duration of production of cytotoxic molecules generated by activated macrophages. Pathogenic microorganisms that are susceptible to respiratory burst products are rapidly killed upon phagocytosis by activated macrophages. Pathogens that are resistant to oxidative insults mounted by the macrophage may be susceptible to subsequent antimicrobial attacks (i.e. nitric oxide, tryptophan degradation, or iron deprivation). Deactivation of the macrophage respiratory burst response would minimize tissue damage in an otherwise futile attempt to eliminate reactive oxygen insensitive pathogens. Moreover, the coordination of temporally arranged antimicrobial responses would maximize the effectiveness of each individual response, allowing macrophages to divert their metabolic energy into a single, extremely potent, but short-lived antimicrobial assault.

Currently, the literature provides limited experimental evidence to support this model of the sequential induction of macrophage antimicrobial killing responses. Studies done in the early to late 1980's demonstrated that mammalian macrophages required stimulation periods of 48-72 hrs with macrophage activating factors, such as IFN-γ or LPS, for

maximal induction of macrophage antimicrobial activity (Nagakawara et al., 1982; Nathan et al., 1983; Stuehr and Marletta, 1987; Werner-Felmayer et al., 1987). The model depicted in Fig 7.2 is dependent on the rapid induction of specific macrophage cytotoxic responses, in particular, the respiratory burst. There is evidence in the literature clearly demonstrating that under certain circumstances mammalian macrophages can undergo rapid induction of respiratory burst activity.

Human monocytes can be primed for enhanced respiratory burst activity after only 30 minutes of stimulation with IFN-γ and TNF-α (Kumaratilake et al., 1993). Lin et al., (1993) have also demonstrated that murine macrophages can undergo rapid priming of their respiratory burst response (i.e. 1-6 hrs) when stimulated with LPS or proteins derived from the pathogen, *Entamoeba histolytica*. It is particularly interesting that this group also observed that macrophages underwent deactivation of their enhanced respiratory burst state after 12 hrs of stimulation with amoebic proteins (Lin et al., 1993).

Recent experiments done in our laboratory (Neumann and Belosevic, unpublished data) demonstrate that murine bone marrow-derived macrophages (BMDM) can also undergo rapid priming of their respiratory burst when stimulated with IFN-γ and/or LPS (Fig 7.1). It is particularly interesting that murine BMDM respiratory burst responses are maximally primed after only 6 hrs of co-stimulation with IFN-γ and LPS, but are subsequently deactivated after 12 hrs of stimulation (Fig. 7.1), an observation identical to that observed by Lin et al. (1993) using amoebic proteins as an activator of macrophages. Moreover, this observation is consistent with Ding et al. (1988), who demonstrated that murine macrophages co-stimulated with IFN-γ and LPS had significantly decreased respiratory burst responses after 48 hrs of stimulation, whereas stimulation of this same population of cells with IFN-γ only resulted in a maximally primed respiratory burst response after 48 hrs of stimulation. However, the conclusion drawn by Ding et al., (1988) was that LPS

inhibited IFN-γ-induced priming of the macrophage respiratory burst response. In light of the data presented in Fig. 7.1, it would appear that co-stimulation of murine macrophages with IFN-γ and LPS accelerates the induction of respiratory burst activity, and that this is followed by a subsequent deactivation of the respiratory burst response. This deactivation of respiratory burst activity coincides with the induction of nitric oxide synthesis (Fig 7.1).

Unfortunately, the majority of experiments aimed at elucidating the mechanisms involved in the regulation of mammalian macrophage antimicrobial responses have been carried out using extensive stimulation periods (i.e. 48 hrs), this protocol based exclusively on the observation that macrophages require these lengthy stimulation periods for induction of antimicrobial activity when stimulated with IFN-γ. However, I believe that the kinetics of macrophage activation are drastically different when comparing macrophages that are stimulated with a single stimulus (i.e. IFN-γ), and those co-stimulated with multiple stimuli (i.e IFN-γ and LPS, Fig 7.1). My results support the hypothesis that vertebrate macrophages undergo accelerated inducible antimicrobial responses when co-stimulated with activating cytokines and pathogen-derived molecules. Macrophages exposed to these two signals become fully activated, in an "all-or-none" response, that ultimately manifests itself as a sequential macrophage antimicrobial response.

It is important to note that this model does not define macrophage activation in the context of how a single macrophage will behave when appropriately stimulated (i.e. a single macrophage does not necessarily have to cycle through all responses). It is clear from the data presented in this thesis that macrophage sub-populations have dramatically different kinetics of activation. For example, the respiratory burst of R2-type goldfish macrophages was primed in response to stimulation with MAF and/or LPS, and that priming potential correlated with the duration of stimulation. Contrary to this, R3-type macrophages were rapidly primed

and subsequently deactivated of their primed state. Moreover, R2-type macrophages produced copious amounts of nitric oxide in response to stimulation with MAF and LPS, whereas R3-type macrophages failed to produce nitric oxide. The sequential induction of macrophage antimicrobial responses was observed only in GMCL and IVDKM cultures; cultures containing mixed populations of R2 and R3-type macrophages. I believe that this "division of labor" among macrophage sub-populations coordinates the sequential induction of macrophage antimicrobial responses.

From my data, it would appear that the R3-type macrophage (i.e. putative monocyte) is a key player in coordinating the sequential induction of macrophage antimicrobial responses. It is this macrophage sub-population that undergoes rapid priming and subsequent deactivation of respiratory burst activity, and also appears to de-prime R2-type macrophage respiratory burst activity. With respect to host defense, monocytes may represent a unique sub-population of macrophage, whose primary purpose may be to regulate cytotoxic responses at sites of inflammation. Monocytes recruited from the circulation to the site of infection can participate in killing by rapidly upregulating respiratory burst activity in response to stimulation with cytokines and/or LPS. However, these cells may limit the duration of reactive oxygen production by subsequently deactivating respiratory burst activity, in order to prevent unnecessary tissue damage incurred by prolonged production of reactive oxygen intermediates by phagocytes. Deactivation of respiratory burst activity may occur as a result of the induction of tryptophan degradation by monocytes. Catabolites of tryptophan would actively scavenge reactive oxygen at the site of inflammation. It is interesting to note that studies aimed at elucidating the molecular mechanisms of macrophage-mediated tryptophan degradation have been described primarily from monocytes or monocyte-derived macrophages (Schmitz et al., 1989; Byrne et al., 1989; Carlin et al., 1989; Paguirigan et al., 1994; Carlin et al., 1995). Monocyte-

mediated tryptophan degradation at the site of inflammation would subsequently play an important role in augmenting production of nitric oxide by tissue macrophages through the secretion of picolinic acid into an hypoxic microenvironment. Although monocytes do not appear to synthesize nitric oxide, they can participate in nitric oxide-mediated killing by using nitrite, produced by tissue macrophages, as a substrate for myeloperoxidase (Eiserich et al., 1998).

Thus, the appropriate activation sequence induced in different macrophage sub-populations coordinates the induction of temporally spaced macrophage antimicrobial responses.

Future Directions

The study of the mechanisms regulating the induction of teleost macrophage activation is still in its infancy. Fish cytokines are not commercially available; thus, elucidation of the cytokine-mediated regulatory mechanisms governing macrophage activation in teleosts, at present, depends on the use of crude cytokine preparations. Consequently, considerable variability is introduced into the experiments, as a result of the multiple macrophage modulators present in crude cytokine preparations. Although crude cytokine preparations make it impossible to attribute a specific action to a specific cytokine, they may mimic more naturally the interactions that occur *in vivo*, where a network of cytokine interactions mediate macrophage immunity. However, it is clear that the primary objective for future research in this area be focused on purifying and cloning cytokines involved in the activation/deactivation of teleost macrophages. Pure cytokine preparations will help reveal the contributions that specific host-derived molecules play in mediating immunity to infectious disease in fish.

The techniques described in this thesis, regarding the establishment of primary macrophage cultures from teleost hematopoeitic tissues, should prove to be an invaluable tool for obtaining sufficient quantities of relatively pure macrophages for experiments. However, the use of crude cell conditioned medium for the establishment of macrophage cultures invariably results in different proportions of macrophage sub-population development, resulting in increased variability between experiments. Purification of the different macrophage sub-populations using flow cytometry is only practical for experiments that require relatively few numbers of cells. Identifying specific macrophage growth factors, and their concomitant isolation and cloning, will greatly facilitate the elucidation of the mechanisms regulating teleost macrophage activation. Primary cultures grown in the presence of purified growth factors may initiate selective development of a specific macrophage sub-population. Moreover, purified growth factors will facilitate research directed at defining the developmental pathways of macrophages in teleosts, an area yet to be explored in fish. In addition, monoclonal antibodies generated against cell determination antigens, found on the various sub-populations of macrophages in IVDKM cultures, would also facilitate the identification of the pathways leading to macrophage development in teleosts. The results described in this thesis set the foundation for this research.

The results and techniques described in this thesis also have practical implications for the aquaculture industry. Currently, vaccine development against infectious fish diseases has focused primarily on identifying pathogen-derived molecules that induce antibody-mediated immunity in fish. It is clear from the mammalian literature that induction of antibody-mediated immunity may be detrimental to the host in many cases. The induction of cell-mediated immunity may be the preferred response for some vaccination strategies, particularly in fish, where cell-mediated immune responses are highly evolved, and antibody-mediated immune responses are primitive. The identification of

pathogen-derived molecules that elicit induction of macrophage activation can be used as a measure of cell-mediated immunity. My research has provided the foundation for accurately assessing cell-mediated immune responses in teleosts.

My research has also prompted the postulation that vertebrate macrophages mount sequential, temporally spaced antimicrobial responses. These observations have a number of important ramifications with respect to our current concept of macrophage-mediated immunity in vertebrates. Most experiments conducted using mammalian macrophages are assayed after 48 hrs of stimulation, and treatment that results in an impaired response is often interpreted as inhibitory with respect to macrophage activation. Clearly, this may not be the case, in that the response being measured may have occurred prior to the time point at which analysis was done. A complete kinetic analysis of the induction of macrophage antimicrobial mechanisms, in response to stimulation with cytokines or pathogen-derived molecules (or combinations thereof), must be characterized. Furthermore, the sequential induction of macrophage activation predicts that certain windows of activation/deactivation ultimately control macrophage function (e.g. a cytokine presented to the macrophage outside its window of stimulation may be perceived as an anergic signal by the macrophage). Thus, a full kinetic analysis of the antimicrobial mechanisms of macrophages induced by host or pathogen-derived molecules must be determined in order to conclusively and objectively define whether a stimulus activates or deactivates a particular macrophage response. Furthermore, it remains to be conclusively determined whether specific sub-populations of macrophages (i.e. monocytes and tissue-derived macrophages) can actively cross-regulate each others functional activities, thereby coordinating the induction of these temporally spaced antimicrobial mechanisms.

Table 7.1. A summary of the spatial and temporal regulatory mechanisms involved in the activation of macrophage antimicrobial responses.

Form of Regulation	Example
spatial regulation	• presence or absence of an activating/ deactivating signal perceived by the macrophage. • dose of the stimulus perceived (i.e. dose response), at a specified time point.
temporal regulation	• specific windows of activation/deactivation (i.e. a cytokine can only exert its effects if the macrophage perceives it at certain time during activation). • duration of production of cytotoxic molecules is restricted to minimize host tissue damage.

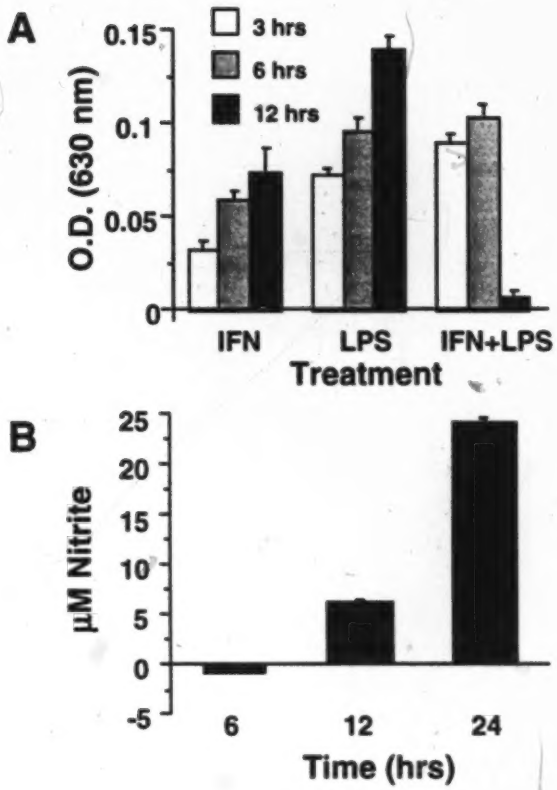

Figure 7.1. Induction of the respiratory burst and nitric oxide production in murine bone marrow-derived macrophages (BMDM). BMDM were established using the methodology described by Guy (1995). BMDM were seeded into wells of a 96 well culture plate ($1x10^5$ macrophages/well) and stimulated with IFN-γ (25 U/ml) and/or LPS (500 ng/ml). PMA-induced respiratory burst activity was measured at the specified time intervals using the NBT assay (A). Nitric oxide production was determined by measuring nitrite in cell supernatants from IFN-γ and LPS stimulated macrophages using the Griess reagents (B). Bars represent the mean ± SEM of twelve cultures in (A) and eight cultures in (B).

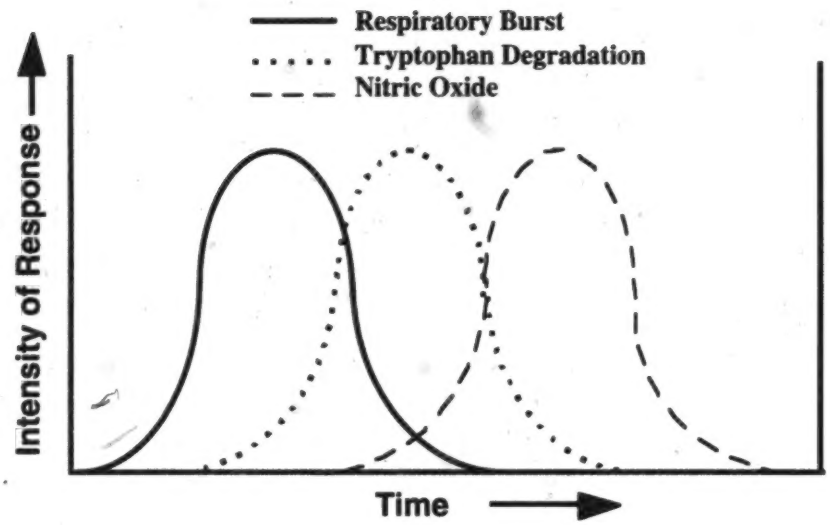

Figure 7.2. A kinetic model of macrophage activation in vertebrates.

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